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NEDD8 Modification of CUL1 Dissociates p120^{CAND1}, an Inhibitor of CUL1-SKP1 Binding and SCF Ligases

Short Article

Jidong Liu,[1] Manabu Furukawa,[1] Tomohiro Matsumoto,[2] and Yue Xiong,[1,3]
[1]Department of Biochemistry and Biophysics
Lineberger Comprehensive Cancer Center
Program in Molecular Biology and Biotechnology
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599
[2]Radiation Biology Center
Kyoto University
Yoshida-Konoe cho
Sakyou ku, Kyoto 606-8501
Japan

Summary

Cullin proteins assemble a large number of RING E3 ubiquitin ligases and regulate various physiological processes. Covalent modification of cullins by the ubiquitin-like protein NEDD8 activates cullin ligases through an as yet undefined mechanism. We show here that p120^{CAND1} selectively binds to unneddylated CUL1 and is dissociated by CUL1 neddylation. CAND1 formed a ternary complex with CUL1 and ROC1. CAND1 dissociated SKP1 from CUL1 and inhibited SCF ligase activity in vitro. Suppression of CAND1 in vivo increased the level of the CUL1-SKP1 complex. We suggest that by restricting SKP1-CUL1 interaction, CAND1 regulated the assembly of productive SCF ubiquitin ligases, allowing a common CUL1-ROC core to be utilized by a large number of SKP1-F box-substrate subcomplexes.

Introduction

Ubiquitination plays an essential and broad role in regulating the stability and activity of many proteins involved in diverse physiological processes. This process requires a cascade of three enzymatic activities for activating (E1), conjugating (E2), and ligating (E3) ubiquitin covalently to a substrate (Hershko and Ciechanover, 1998). The E3 ubiquitin ligases have two distinct functions: one for recruiting the substrates and the other for catalyzing isopeptide bond formation (ubiquitin ligation). Two major families of E3 ligases have been described: the HECT domain family that is defined by its homology to E6-associated protein (E6AP) carboxyl terminus and the RING family that contains either an intrinsic RING finger domain or an associated RING finger protein subunit essential for the ubiquitin ligase activity (Zachariae and Nasmyth, 1999; Deshaies, 1999; Jackson et al., 2000).

One of the best characterized RING E3 ligases is the ROC1-SKP1-cullin1/Cdc53-F box protein (SCF) complex that targets the ubiquitination of various proteins involved in cell cycle control and signal transduction. ROC1-SCF consists of a scaffold protein, Cdc53/cullin 1, which can bind simultaneously with an adaptor molecule, SKP1, via an N-terminal domain and with a small RING finger protein, ROC1 (also known as Hrt1 and Rbx1), via a C-terminal domain. Through SKP1, CUL1 associates with an F box protein that in turn binds phosphorylated substrate (Deshaies, 1999; Jackson et al., 2000). The CUL1 component of SCF E3 ligase belongs to an evolutionarily conserved family of proteins known as cullins that contains at least six related members in human. Assembly of the multisubunit complex is thought to provide the specificity and versatility to target various proteins for ubiquitination by the same catalytic core. The mechanisms governing the assembly of productive SCF complex are not known.

Most, if not all, cullin proteins examined to date are modified by the ubiquitin-like protein NEDD8/Rub1 (Lammer et al., 1998; Liakopoulos et al., 1998; Osaka et al., 1998; Hori et al., 1999). NEDD8 modification, although not essential in budding yeast (Lammer et al., 1998; Liakopoulos et al., 1998), substantially enhances the ligase activity of SCF (Furukawa et al., 2000; Podust et al., 2000; Read et al., 2000; Wu et al., 2000) and is required for cell viability in fission yeast (Osaka et al., 2000) and mouse embryonic development (Tateishi et al., 2001). The mechanism of NEDD8 modification in activating CUL1-associated ubiquitin ligase activity is unknown. The current study is directed toward this issue.

Results

Wild-Type CUL1 Associates with p120^{CAND1}

We examined protein complexes containing wild-type and CUL1 mutant that are defective in both conjugation with NEDD8 and in nuclear accumulation by alterations in the C terminus (K720A/L756A/I757A) (Furukawa et al., 2000). A protein with an apparent molecular weight of 120 kDa (p120) was identified that associated with wild-type CUL1 but was not detected in the CUL1 (K720A/L756A/I757A) immunocomplex (Figure 1A). This protein, which we have named CAND1 (for cullin-associated and neddylation-dissociated), was immunopurified. Amino acid sequences of five peptides of p120^{CAND1} were determined, all matching with a previously described protein, TIP120A. CAND1 was reported in association with TBP (Yogosawa et al., 1996), several proteasomal ATPases of the 19S regulatory particle (Makino et al., 1999), and KIAA10 HECT domain E3 ligase complex (You and Pickart, 2001). The physiological and biochemical relevance of those interactions is unclear. CAND1 has a highly related homolog in the mammalian genome, CAND2/TIP120B, which is specifically expressed in muscle tissues (Aoki et al., 1999). CAND is a single gene in fruit fly, *Arabidopsis*, *Caenorhabditis elegans*, and possibly in fission yeast, but is notably absent in budding yeast. Human CAND proteins contain 25 HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, TOR1) motifs that distribute continuously throughout the entire sequence (Neuwald and Hirano, 2000). HEAT motifs,

[3]Correspondence: yxiong@email.unc.edu

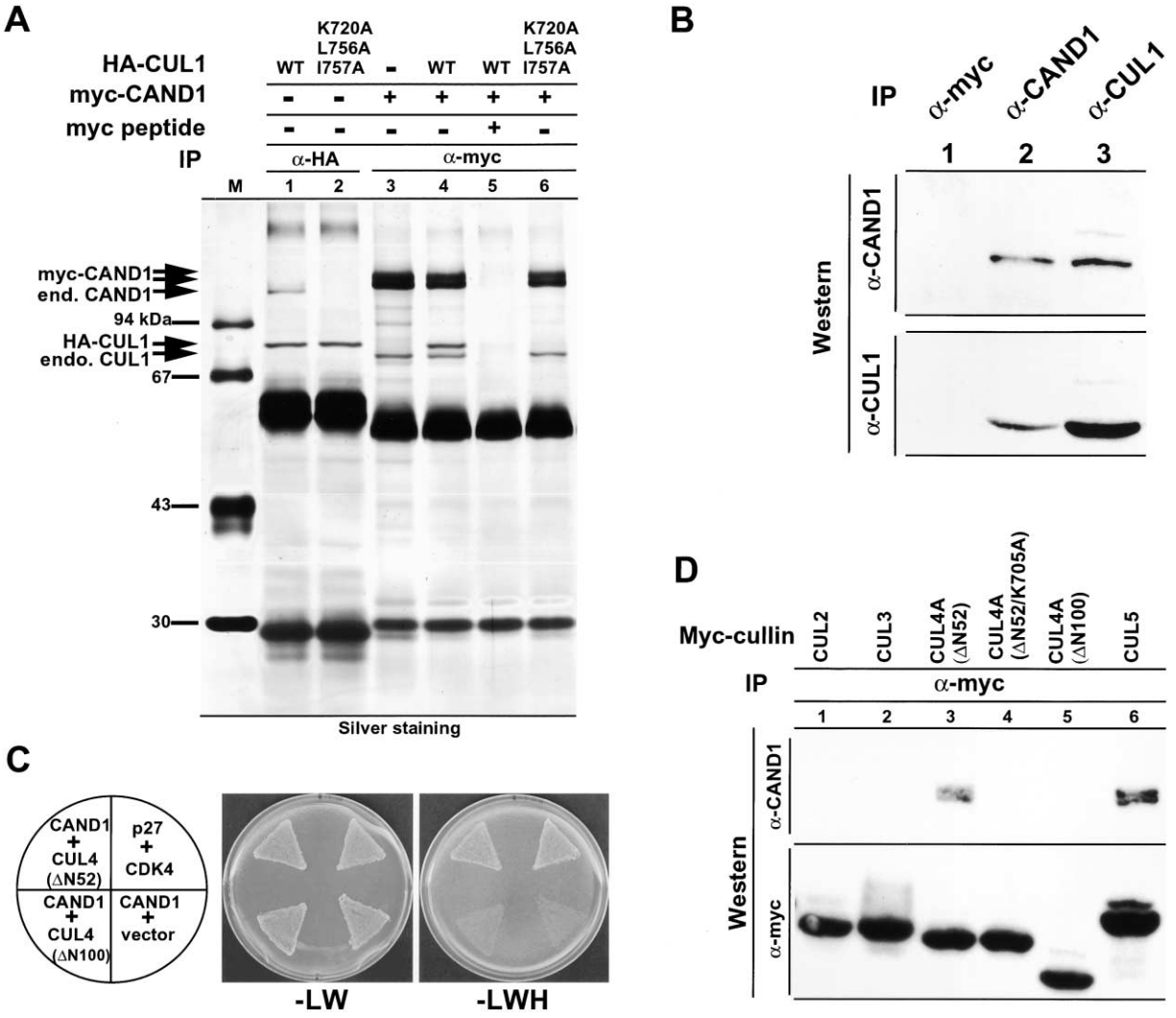

Figure 1. Identification of CAND1-CUL1 Association

(A) 293T cells were transfected with the indicated plasmids expressing wild-type or mutant CUL1 proteins in combination with CAND1. Lysates prepared from transfected cells were immunoprecipitated with the indicated antibodies and resolved by SDS-PAGE, followed by silver staining.

(B) CAND1 associates with CUL1 in vivo. 293T cells were lysed and immunoprecipitated with antibodies recognizing CAND1, CUL1 or with a control myc antibody. In vivo association of CAND1 and CUL1 was determined by reciprocal IP-Western.

(C) CAND1 interacts with CUL4A in yeast two-hybrid assay. CUL4A (ΔN52) contains 48 NH_2-terminal residues in addition to that of the longest published clone (Q13619) CUL4A (ΔN100) but is missing 52 residues based on our very recent characterization of EST clones and human genome sequence.

(D) CAND1 interacts with other cullins. Plasmids expressing various human cullins were transfected into 293T cells, and their interaction with endogenous CAND1 was examined by IP-Western.

composed of a pair of antiparallel α helices, are degenerate in sequence and vary in length between 30 and 45 residues (Andrade and Bork, 1995). HEAT motifs are thought to be involved in protein-protein interaction and to serve as flexible scaffolding on which other proteins can assemble.

CAND1 Associates with Multiple Cullins

Ectopically expressed myc-tagged CAND1 associated with endogenous CUL1 and ectopically expressed the wild-type CUL1 but not CUL1 (K720A/L756A/I757A) mutant (Figure 1A). Addition of a molar excess of competing myc antigen peptide to the cell extract blocked the precipitation of myc-CAND1 and CUL1 proteins (lane 5). An in vivo association between endogenous CAND1 and CUL1 was readily detected in untransfected 293T cells (Figure 1B), providing further evidence supporting their interaction under physiological conditions. We carried out a yeast two-hybrid screen to search for CAND1-interacting proteins. Of an estimated 1×10^6 transformants screened, only one confirmed positive clone was identified, which encoded a truncated human CUL4A protein missing the N-terminal 52 residues (Figure 1C). We further examined the interaction between CAND1 and other cullin family members in the transfected 293T cells and found that CAND1, in addition to CUL1 and CUL4A, could also interact with CUL5 (Figure 1D). Mutation of the NEDD8 conjugation site (lane 4) or

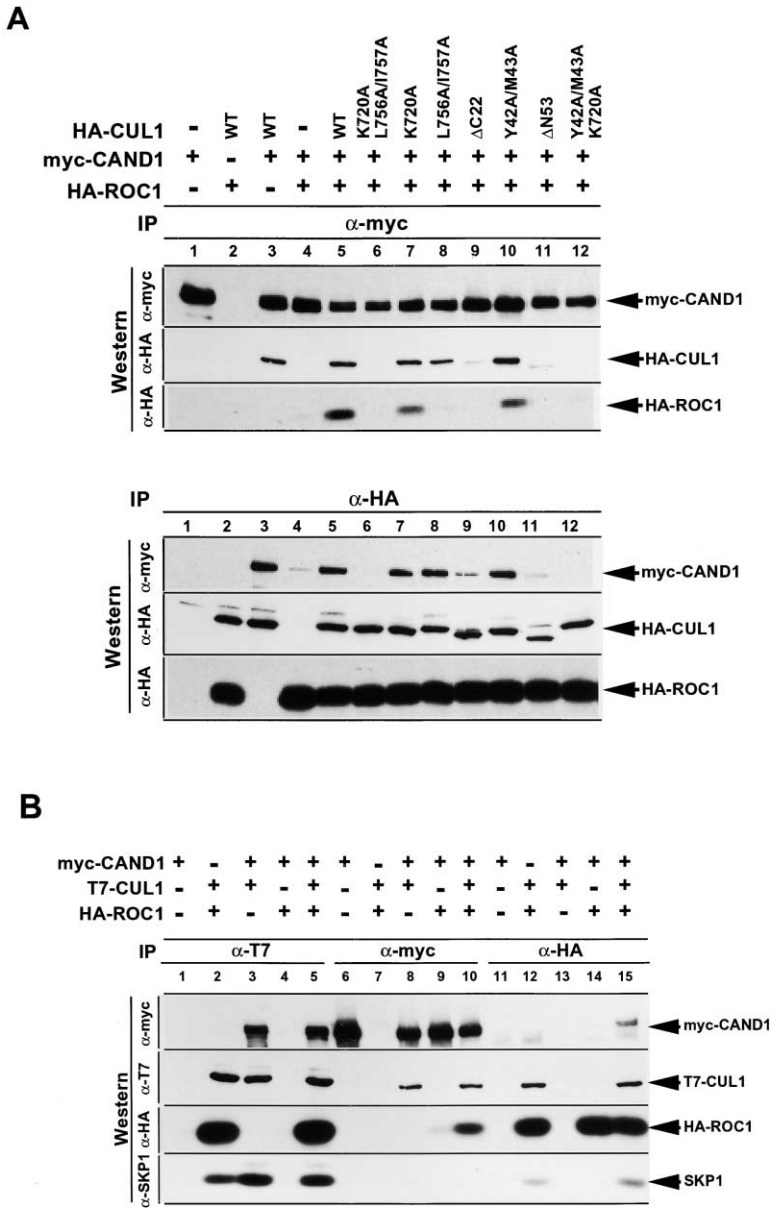

Figure 2. CAND1 Forms a Ternary Complex with CUL1-ROC1

(A) Mapping sequences in CUL1 important for interacting with CAND1. 293T cells were cotransfected with plasmids expressing myc-CAND1 and wild-type or mutant CUL1 proteins. CAND1-CUL1 binding was determined by IP-Western.

(B) CAND1 forms a ternary complex with CUL1-ROC1. 293T cells were cotransfected with plasmids expressing the indicated proteins. Protein complex formation between CAND1, ROC1, CUL1, and SKP1 was examined by reciprocal IP-Western.

deletion of NH_2-terminal 100 residues of CUL4A disrupted its association with CAND1 (lane 5). Hence, CAND1 can associate with multiple cullins. CAND1-cullin association requires the NH_2-terminal sequences of cullins and could potentially be affected by the NEDD8 modification of cullins.

CAND1-CUL1 Forms a Ternary Complex with ROC1

Deletions of either an NH_2-terminal (520 residues) or a COOH-terminal (298 residues) sequence from CAND1 disrupted its binding with CUL1 (data not shown), suggesting that binding with CUL1 may involve both ends of CAND1 protein. Conversely, deletion of either 53 residues from the NH_2-terminus (ΔN53) or 22 residues from the COOH terminus (ΔC22) of CUL1 abolished its ability to associate with CAND1 (Figure 2A). Mutants CUL1 (K720A), CUL1 (Y42A/M43A), and CUL1 (L756A/I757A) were capable of binding with CAND1 when CAND1 was overexpressed. The combination of K720A with the double mutations at either the NH_2 or COOH terminus of CUL1, however, completely abolished CUL1-CAND1 interaction even when both proteins were highly expressed. These results suggest that multiple domains in CUL1 are involved in binding with CAND1, including the N-terminal domain that overlaps with Skp1 binding, the C-terminal region involved in nuclear localization/accumulation of CUL1, Lys-720, and the sequence surrounding Lys-720.

We next examined the CUL1-CAND1 interaction with respect to two other CUL1-interacting proteins, ROC1 and SKP1. CAND1 was detected in the ROC1 immunocomplex (Figure 2B, lane 15), suggesting that ROC1 and CAND1 are not competing for binding with CUL1. In the absence of cotransfection of CUL1, CAND1 and ROC1 were not detected to associate with each other (lanes 9 and 14), indicating that ROC1-CAND1 interaction is

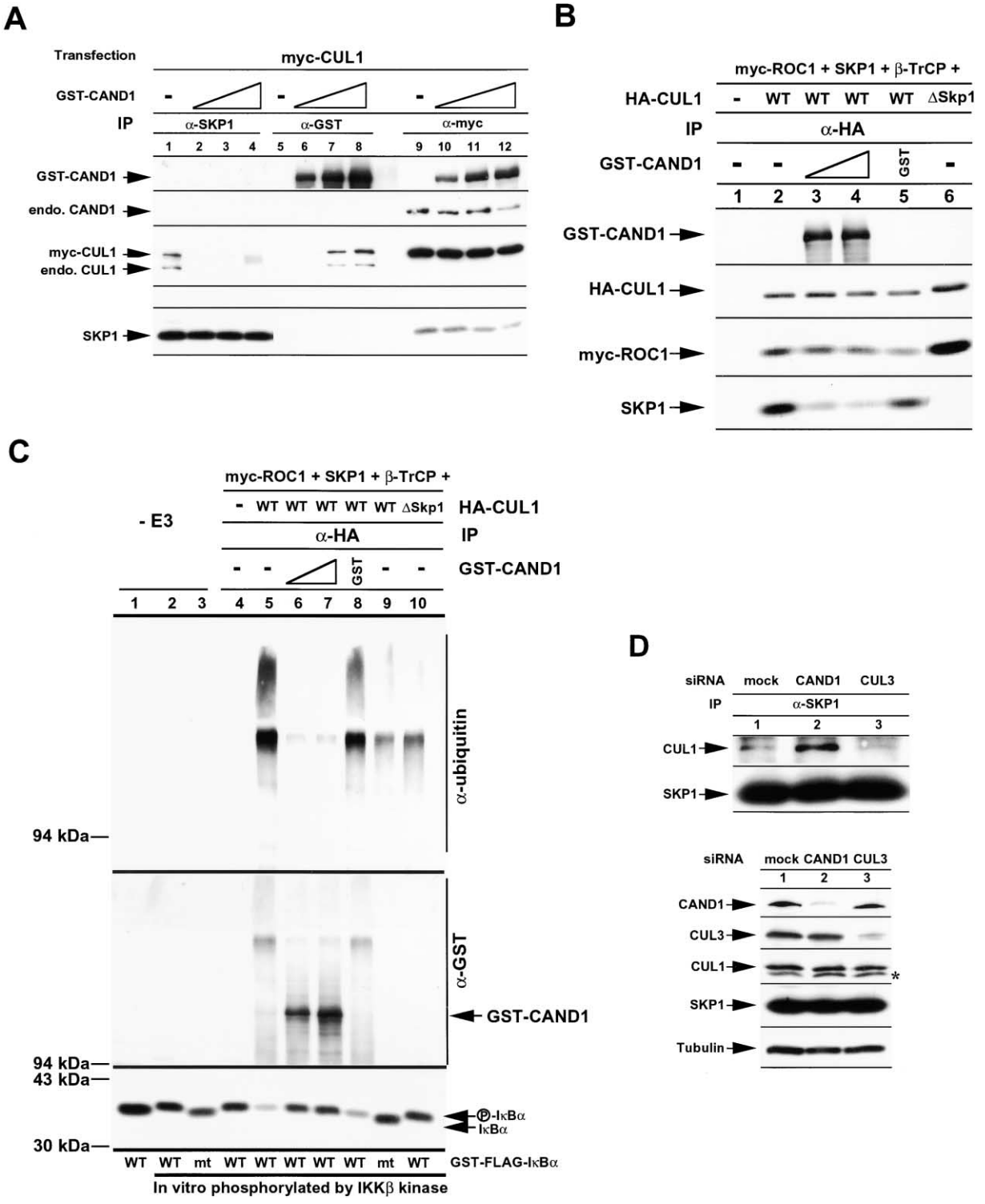

Figure 3. CAND1 Inhibits SKP1-CUL1 Interaction and SCF Ubiquitin Ligase Activity

(A) CAND1 blocks binding of SKP1 with CUL1. Lysates were prepared from 293T cells transfected with a plasmid expressing myc-CUL1 and incubated with increasing amounts of purified GST-CAND1 fusion protein. Mixtures were then immunoprecipitated with the indicated antibodies. SKP1-CUL1 and CAND1-CUL1 complexes were examined by immunoblotting.

(B) CAND1 dissociates SKP1 from preassembled SKP1-CUL1. $SCF^{\beta\text{-TrCP}}$ complex was immunoprecipitated using HA antibody from cells cotransfected with HA-CUL1, myc-ROC1, SKP1, and β-TrCP, and incubated with increasing amounts of purified GST-CAND1. After incubation, HA-CUL1 immunoprecipitates were washed, and CUL1-associated proteins were examined by immunoblotting.

(C) CAND1 dissociates substrate-targeting molecules from CUL1 and inhibits ubiquitination of $SCF^{\beta\text{-TrCP}}$ substrate in vitro. Purified wild-type (WT) and phosphorylation deficient (mt) GST-FLAG-IκBα (1–54) protein was phosphorylated with $IKK\beta^{S177E/S181E}$ kinase and then subjected to

mediated by CUL1 rather than directly with each other. In contrast, although both CAND1 and SKP1 can be detected in the CUL1 or ROC1 immunocomplexes, no SKP1 was detected in the CAND1 complex (Figure 2B, lanes 6–10) whether or not ROC1 was coexpressed, suggesting that CAND1 and SKP1 formed different complexes with CUL1 and may interact with CUL1 competitively (see below). This idea is consistent with the observation that deletion of an NH_2-terminal sequence in CUL1 (ΔN53) abolished its binding with both SKP1 and CAND1.

CAND1 Inhibits SKP1 Binding to CUL1

To further determine CUL1's interaction mode with SKP1 and CAND1, we incubated increasing amounts of GST-CAND1 fusion protein purified from bacteria with a lysate derived from cells transfected with a plasmid expressing myc-CUL1 and analyzed CUL1-SKP1 and CUL1-CAND1 association by reciprocal IP-Western (Figure 3A). Endogenous SKP1 formed a complex with both the endogenous CUL1 and ectopically expressed myc-CUL1 (lanes 1 and 9). The addition of an increased amount of GST-CAND1 resulted in the formation of an increased amount of CUL1/GST-CAND1 complexes. Correlated with this increase was a decrease of CUL1-associated SKP1 as well as CUL1-associated endogenous CAND1.

To examine whether CAND1 could dissociate preassembled CUL1-SKP1-F box complex, SCF$^{\beta\text{-TrCP}}$ complex was incubated with increasing amounts of purified GST-CAND1 or control GST protein. After incubation, HA-CUL1 immunoprecipitates were washed and divided into two parts: one for examining CUL1-SKP1 and CUL1-ROC association (Figure 3B) and one for assaying in vitro IκBα ubiquitination (Figure 3C). HA-CUL1 formed readily detectable complexes with both ectopically expressed myc-ROC and SKP1 (Figure 3B, lane 2). Incubation with GST-CAND1, like the mutation at two residues in CUL1 critical for SKP1 binding (Y42A/M43A, lane 6), dissociated SKP1 from the CUL1 complex but had no effect on CUL1-ROC1 binding (lanes 3 and 4).

SCF$^{\beta\text{-TrCP}}$ immunocomplex efficiently ubiquitinated IκBα, converting nearly all IKKβ-phosphorylated GST-FLAG-IκBα into high molecular weight conjugates that were detected by both anti-ubiquitin and anti-GST antibodies (Figure 3C). Omitting HA-CUL1 from transfection, disrupting CUL1-SKP1 binding, or mutating IKKβ phosphorylation sites in IκBα all abolished IκBα ubiquitination, confirming the specificity of SCF$^{\beta\text{-TrCP}}$ dependent in vitro IκBα ubiquitination. Addition of purified GST-CAND1 substantially inhibited IκBα ubiquitination. Thus, CAND1 dissociates SKP1 from CUL1 and thereby inhibits its SCF ligase activity. Mutations destroying IKKβ-phosphorylation sites abolished polyubiquitination of IκBα but not ROC1-mediated autoubiquitination as detected by anti-ubiquitin antibody (lane 9). In the same assay, the level of polyubiquitin ladders was significantly reduced when purified GST-CAND1 was added to the reaction (lanes 6 and 7). This data suggested CAND1 might also interfere with the ROC1-mediated autoubiquitination.

To probe the in vivo function of CAND1 protein in regulating CUL1-SKP1 association and SCF activities, we knocked down the expression of CAND1 protein in HeLa cells by RNA interference (RNAi). The level of CAND1 protein was substantially reduced (>90%) by transfection of siRNA oligonucleotides targeting the CAND1 but was not significantly affected by mock transfection or transfection of siRNA oligonucleotides targeting CUL3 (Figure 3D). Reduced CAND1 expression did not affect the level of SKP1 or CUL1 but significantly increased CUL1-SKP1 association, confirming the function of CAND1 in negative regulation of CUL1-SKP1 binding in vivo.

CAND1 Selectively Associates with the Unneddylated Form of CUL1

In all CAND1 complexes that have been examined, only the unmodified form of CUL1 was seen in association with CAND1; the NEDD8-modified form of CUL1 was never detected (e.g., Figure 2), suggesting that NEDD8 conjugation of CUL1 is not required for binding with CAND1. In vivo, only a small portion of CUL1 is conjugated with NEDD8, making it difficult to determine whether lack of CUL1-NEDD8 in the CAND1 complex is due to the low level of CUL1-NEDD8 conjugate or a selective interaction of CAND1 with the unmodified form of CUL1. To address this issue, we examined the interaction between CAND1 and a hyperneddylation CUL1 (R473A/L474A) mutant (Lammer et al., 1998; Patton et al., 1998). Ectopically expressed CUL1 (R473A/L474A) produced both unmodified CUL1 and NEDD8-modified CUL1 in similar ratio, but only the unmodified form was detected in the CAND1 immunocomplex (Figure 4A). To exclude the possibility that lack of CAND1's association with neddylated CUL1 may be caused by different subcellular localizations of the two proteins, we incubated purified GST-CAND1 fusion protein with extracts derived from cells transfected with plasmids expressing ROC1 and either wild-type or CUL1 (R473A/L474A) mutant. Addition of gluthathione agarose beads precipitated GST-CAND1 and the unmodified form of CUL1 but not the slower migrating CUL1-NEDD8 (Figure 4B).

NEDD8 Modification of CUL1 Dissociates CAND1

To determine how NEDD8 modification affects CUL1's interaction with CAND1, we incubated preassembled CAND1-CUL1 complexes with NEDD8-conjugating enzymes followed by an examination of CAND1-CUL1 association (Figure 4C). Myc-CAND1/HA-CUL1 complexes were immobilized onto protein A/G beads and incubated with NEDD8 activating and conjugating enzymes. Following the incubation the mixture was resolved by SDS-

ubiquitin ligation assay by the indicated CUL1 immunocomplexes. After termination of reaction, mixtures were resolved by SDS-PAGE followed by immunoblotting using antibodies specific to FLAG, GST, and ubiquitin.

(D) Suppression of CAND1 in vivo increased SKP1-CUL1 interaction. HeLa cells were transfected with siRNA oligonucleotides targeting CAND1 or CUL3. The expression of various proteins was determined by immunoblotting. The formation of the SKP1-CUL1 complex was determined by IP-Western. An asterisk indicates a nonspecific protein detected by the CUL1 antibody.

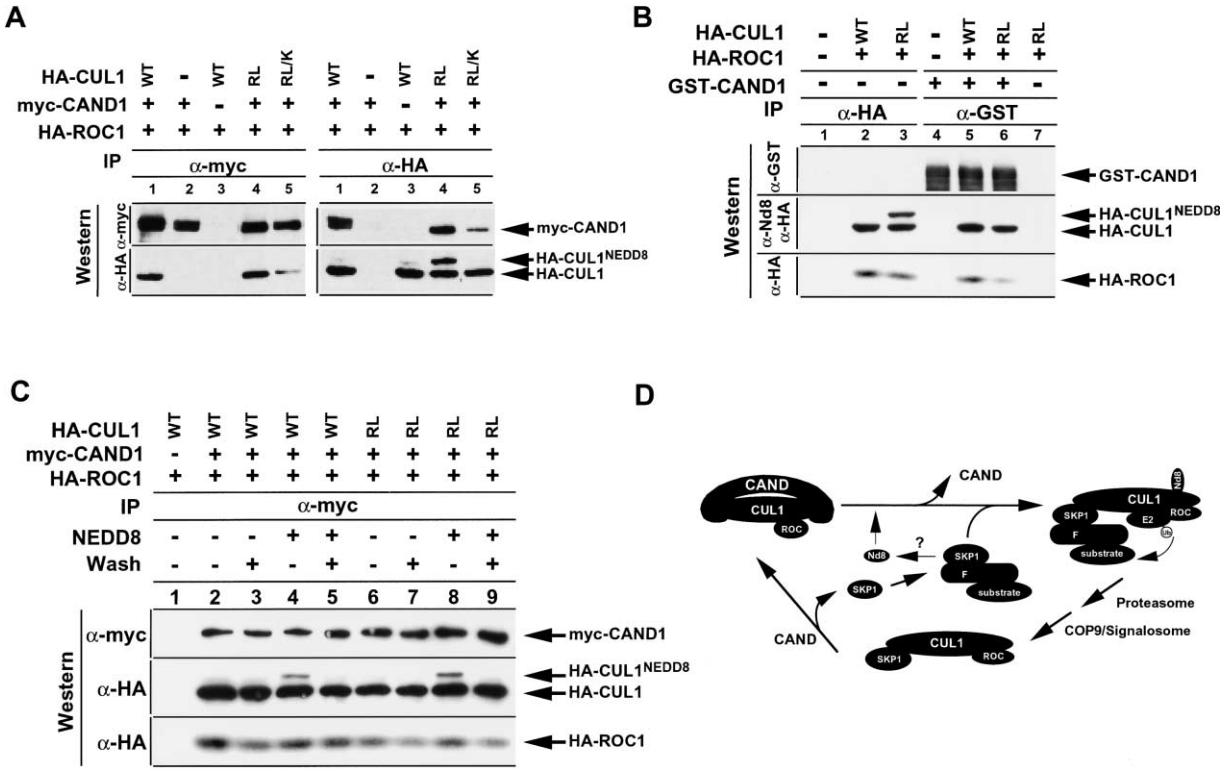

Figure 4. NEDD8 Modification Prevents CUL1-CAND1 Association

(A) CAND1 selectively associated with an unmodified form of CUL1 in vivo. 293T cells were transfected with the indicated plasmids. CUL1-CAND1 associations were examined by IP-Western.

(B) CAND1 selectively binds with the unmodified form of CUL1 in vitro. Lysates were prepared from 293T cells transfected with plasmids expressing the indicated proteins and incubated with purified GST-CAND1 protein. Mixtures were then immunoprecipitated with the indicated antibody, and CUL1-CAND1 associations were examined by immunoblotting.

(C) NEDD8 modification of CUL1-dissociated CAND1. CUL1-CAND1 complexes were immunopurified using myc antibody from cells transfected with plasmids expressing the indicated proteins and incubated with NEDD8-conjugating enzymes. The reaction mixtures were subjected to SDS-PAGE either directly or after washing.

(D) Schematic representation of CAND1 function. CAND protein is proposed to fold into an extended structure and interacts with multiple domains in CUL1. CAND forms a ternary complex with CUL1 and ROC1 and prevents SKP1 from binding to CUL1. Signal-dependent formation of SKP1-F box protein-substrate complex is suggested to trigger neddylation of CUL1 (denoted by a question mark), leading to the dissociation of CAND1 and the assembly of productive ROC-SCF E3 ligase.

PAGE either directly or after washing with NP40 buffer. Incubation with NEDD8-conjugating enzymes resulted in the appearance of a slower migrating NEDD8-modified form of both wild-type and R473A/L474A mutant CUL1 (lanes 4 and 8), suggesting that NEDD8-conjugating enzymes were capable of accessing and modifying CAND1-associated CUL1. Washing the reaction mixture prior to SDS-PAGE completely removed the NEDD8-modified CUL1 (lanes 5 and 9), indicating that NEDD8 conjugation prevents or dissociates CUL1 from binding with CAND1.

Discussion

Four lines of evidence support CAND1 as a negative regulator of SKP1-CUL1 binding. First, CAND1 binds to a region in CUL1 that overlaps with the SKP1 binding region (Figure 2). Second, CAND1 does not coexist with SKP1 in the same CUL1 complex in vivo (Figure 2) and dissociates SKP1 from CUL1 in vitro (Figure 3). Third, loss of CAND1 by RNAi increases SKP1-CUL1 complex in vivo (Figure 3D). Finally, CAND1 inhibits in vitro IκBα ubiquitination by $SCF^{\beta\text{-}TrCP}$ (Figure 3C). We propose that CAND proteins confine catalytically active CUL1-ROC in a functionally immature state and prevent SKP1 from indiscriminately binding to CUL1-ROC. Neddylation of the CUL1, conceivably coupled with or triggered by the formation of SKP1-F box protein-substrate complex, dissociates CAND, resulting in the assembly of an active ubiquitin ligase and subsequent substrate ubiquitination.

With approximately 50 genes encoding F box proteins in mammals, the incorporation of individual F box proteins into the active CUL1 ligase complex has to be tightly controlled. CUL1 is expressed at a much lower concentration than SKP1 and F box proteins, indicating that it is a rate-limiting factor in the assembly of a large number of SCF ligases. While there is a clear necessity for tight control over the dynamic and destructive ubiquitin ligase complexes, all the previously characterized subunits of the SCF-ROC ligases (CUL1, SKP1, F box, and ROC) are involved in either catalytic activity or sub-

strate recruitment. NEDD8 modification of CUL1 was the only factor known to play a role in regulating cullin ligases, but its mechanism remained elusive. CAND1 functions as a negative regulator of SCF activity and is regulated by neddylation on CUL1. By regulating the assembly of SCF ubiquitin ligases, CAND1 allowed a large number of SKP1-F box-substrate subcomplexes to be utilized by the same CUL1-ROC core (Figure 4D). CAND1 could also interact with and similarly regulate other cullins.

The NEDD8 pathway is essential for fission yeast cell viability and mouse development (Osaka et al., 2000; Tateishi et al., 2001). Loss-of-function of NEDD8-activating enzyme APP-BP1 in ts41 Chinese hamster cells uncouples DNA replication and mitosis, leading to endoduplication (Chen et al., 2000; Handeli and Weintraub, 1992; Hirschberg and Marcus, 1982). The physiological significance of cullin neddylation was further underscored by the finding that the COP9/signalosome, an eight subunit complex that had previously been implicated in a wide range of physiological processes ranging from cell cycle control in fission yeast and photomorphogenesis in *Arabidopsis* to signal transduction in mammalian cells (Schwechheimer and Deng, 2001) contains a function required for cleaving CUL1-NEDD8 conjugates (Lyapina et al., 2001). We have demonstrated that CAND1 selectively associated with unneddylated CUL1 and was dissociated from CUL1 by NEDD8 conjugation. These results provide a mechanistic explanation—dissociating an inhibitor of SCF assembly—for the requirement of the NEDD8 pathway in vivo.

Experimental Procedures

Plasmids, Cell Culture, and Cell Transfection

Plasmids expressing various wild-type and mutant human cullins and ROC1 were described previously (Michel and Xiong, 1998; Ohta et al., 1999; Furukawa et al., 2000). Yeast two-hybrid procedures were described in Michel and Xiong (1998) and Ohta et al. (1999). Full-length human CAND1 cDNA was constructed from EST clones AI034263 and KIAA0829. Mutations were introduced by site-directed mutagenesis using the QuikChange Kit (Stratagene) and verified by DNA sequencing. Human 293T and HeLa cells were cultured in DMEM (10% FBS) in a 37°C incubator with 5% CO_2. Cell transfections were carried out using calcium-phosphate buffer. For each transfection, 2.5 or 15 μg of total DNA was used for 6-well or 100 mm dishes, respectively. Procedures for immunoprecipitation and immunoblotting have been described previously (Jenkins and Xiong, 1995). Antibodies to human CUL1, SKP1 were previously described (Michel and Xiong, 1998; Ohta et al., 1999). Antibodies to HA (12CA5, Boehringer-Mannheim), to myc (9E10, NeoMarker), to T7 (Novagen), to FLAG (M2, Sigma), to CUL1 (sc-8552, Santa Cruz), to CAND1/TIP120A (sc-10672, Santa Cruz), and to NEDD8 (Zymed) were purchased commercially.

Purification of CAND1 Protein

To purify CUL1-associated p120, ten 100 mm plates of 293T cells were transfected with a plasmid expressing HA-tagged wild-type CUL1. Thirty-six hours after transfection, cells were lysed with an NP-40 lysis buffer, and lysates were pooled (estimated 100 mg total) and immunoprecipitated with 150 μg of anti-HA antibody overnight at 4°C with rotation. The HA-CUL1 complex was precipitated and resolved by 10% SDS-PAGE. The gel was incubated at room temperature twice for 15 min each in 50% methanol (HPLC grade), 15 min in 5% methanol, and 15 min in 32 μM fresh DTT solution; silver stained (1 mg/ml of $AgNO_3$) for 15 min; and developed in solution containing 0.05% formaldehyde and 30 mg/ml Na_2CO_3 for 2 min. The protein band corresponding to the 120 kDa (estimated 15 μg or 120 pmol) was cut from the gel, minced into smaller pieces, digested with 50 ng/ml lysylendopeptidase, extracted by acetonitrile, and separated by reverse-phase high pressure liquid chromatography on a Hewlett Packard 1100 HPLC system using a C18 column (1 mm × 250 mm, Vydac). Amino acid sequences of individual peptides collected from HPLC were determined on an automated ABI microsequencer or by mass spectrometric analysis. Amino acid sequences of five peptides were determined (KDFRFIATNDLM TELQK, KTVSPALISRFK, KADVFHAYLS, KVIRPLDQPSSFDATPYIK, and KSPLMSTFQSQISSNPE).

In Vitro Ubiquitin and NEDD8 Ligation Assays

In vitro IκBα ubiquitination assays were carried as described (Ohta et al., 1999; Furukawa et al., 2000, 2002). To prepare substrate for in vitro NEDD8 ligation assay, myc-CAND1/HA-CUL1/ROC1 complex was obtained by immunoprecipitation using anti-myc antibody from 293T cells transfected with plasmids expressing both proteins. CAND1-CUL1 complexes immobilized on protein A/G beads were incubated with the NEDD8 conjugation enzyme mixture containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM ATP, 0.6 mM DTT, 0.1 mg/ml bovine serum albumin, 5 ng of NEDD8-activating E1 (APP-BP1/Uba3), 0.1 μg of NEDD8 E2 (GST-Ubc12), and 0.6 μg of NEDD8. The mixture was incubated at 37°C for 1 hr. After NEDD8 conjugation, reaction mixtures were added to an equal volume of SDS sample buffer either directly or after washing the beads twice with buffer containing 0.1% NP40 and resolved by SDS-PAGE followed by immunoblotting analysis.

RNA Interference

siRNA oligonucleotides targeting nucleotides 82 to 102 relative to the translation initiation codon of human CAND1, as well as control siRNA oligonucleotides to CUL3, were synthesized at Dharmacon (Lafayette, CO) as purified and annealed duplexes form. Forty-eight microliters OPTI-MEM medium was mixed with 12 μl oligofectamine reagent for 10 min and incubated with a mixture containing 12 μl siRNA and 200 μl OPTI-MEM medium for 20 min at room temperature. An additional 128 μl of OPTI-MEM medium was added to the mixture, and the entire 400 μl was added to HeLa cells cultured on a 6-well plate at 30%–40% confluency. The cells were transfected three times every 24 hr and lysed 24 hr after the last transfection.

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