CAND1 enhances deneddylation of CUL1 by COP9 signalosome

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Abstract

Cullin-RING ligases (CRLs) regulate diverse cellular functions such as cell cycle progression and cytokine signaling by ubiquitinating key regulatory proteins. The activity of CRLs is controlled by Nedd8 modification of the cullin subunits. Recent reports have suggested that CAND1, which specifically binds to unmodified CUL1 but not to neddylated one, is required for the in vivo function of SCFs, the CUL1-containing CRLs. We show here that CAND1 and COP9 signalosome (CSN), the major deneddylase of cullins, bind to unneddylated CUL1 in a mutually exclusive way. The suppression of CAND1 expression by small inhibitory RNA enhanced the interaction between CUL1 and CSN, suggesting that CAND1 inhibited the binding of CSN to CUL1. We found that the binding of CSN to CUL1 required the four helix bundle in CUL1 C-terminal domain, which was wrapped around by CAND1 in the CAND1-CUL1-Rbx1 complex. CAND1 greatly facilitated CSN-mediated deneddylation of CUL1 in vitro, which was dependent on its binding to CUL1. Our data suggest that enhancement of CSN-mediated deneddylation by CAND1 may contribute to its function as a positive regulator of SCFs in vivo.
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Cullin-RING ligases (CRLs) are a family of ubiquitin ligases that contain a member of cullin family proteins and the RING finger protein Rbx1 (also known as ROC1 and Hrt1) or Rbx2, which together form the integral core of the enzymes [1–4]. Cullins are linked to targeted substrates by specific adaptor proteins and an array of substrate-recognizing subunits, while Rbx1 or Rbx2 interacts with the cognate ubiquitin conjugating enzyme [5]. In SCF complexes, the prototype of CRLs, the N-terminus of CUL1 interacts with the adaptor Skp1, which, in turn, recruits F-box proteins containing a variable protein–protein interaction domain that directly binds substrates [6–8]. CRLs with other cullin proteins appear to share an analogous modular architecture. There exist a large number of F-box, SOCS-box, VHL-box, and BTB domain proteins presumed to form complexes with distinct cullins, which makes CRLs the largest class of E3 ubiquitin ligases [9–13].

Activities of CRLs are regulated by modification of cullin subunits with the ubiquitin-like protein Nedd8 [14]. The Nedd8 conjugation pathway has been reported to be essential for cell viability in fission yeast [15] and for the early development in *Caenorhabditis elegans*, *Arabidopsis*, and mice [16–18]. Conjugation of Nedd8 to CUL1 increases the activity of SCF complexes and enhances E2-ubiquitin recruitment to SCF ubiquitin ligases in vitro [19–22]. Defects in the neddylation pathway lead to substrate accumulation, consistent with the notion that neddylation of cullins positively regulates CRLs [23]. Deconjugation of Nedd8 is mediated

by the COP9 signalosome (CSN), a conserved multi-protein complex composed of eight subunits [24,25]. Genetic studies show that mutations in CSN subunits which lead to constitutive neddylation of cullins cause substrates to accumulate, indicating that deneddylation by CSN also positively regulates the function of CRLs [24,26]. These studies have suggested that the cycles of neddylation and deneddylation may be needed to sustain optimal CRL activities.

In searching for proteins regulating CRLs, we and others have identified CAND1/TIP120A as a cullin-interacting protein [27–30]. CAND1 forms a complex with the CUL1-Rbx1 core and inhibits the activity of SCFs in vitro by competing with Skp1 and F-box proteins for CUL1 binding. CAND1 binding to the unmodified CUL1 blocks Rbx1-mediated neddylation of CUL1 [31,32]. Once neddylated, CUL1 can no longer bind to CAND1. The crystal structure of the CAND1-Cul1-Rbx1 complex reveals that CAND1 occupies the Skp1 binding site of CUL1 and the CUL1 neddylation site is buried by CAND1. Genetic studies with *Arabidopsis thaliana* further show that plant CAND1 is required for normal function of SCFs, suggesting that CAND1 is a positive regulator of SCF function [33,34]. Collectively, these studies support the idea that association/dissociation of CAND1 coupled to neddylation/deneddylation of cullins plays an important role in assembly and disassembly of functional SCFs and other CRLs. It remains to be determined, however, how cycles of assembly and disassembly of functional CRLs operate. In this report, we show that the binding of CAND1 and CSN to CUL1 is mutually exclusive and present evidence that CAND1 facilitates CSN-mediated CUL1 deneddylation.

Materials and methods

Plasmids. The cDNAs encoding human CSN1 (Accession No. U20285), CSN2 (BC012629), CSN3 (BC001891), CSN4 (BC004302), CSN5 (BC001187), CSN6 (BC002520), CSN7A (BC011789), and CSN8 (BC003095) were obtained from the Medical Research Council (United Kingdom) gene service. To construct plasmids for the expression of N-terminally FLAG- or HA-tagged proteins, cDNAs were amplified by PCR with appropriate primers and ligated into pYR vectors [29] or pcDNA3.1 (Invitrogen). Expression constructs of human FLAG-CUL1 and its deletion mutants were previously described [31].

Stable cell lines, protein complex purification, and protein identification by mass spectrometry. HeLa Tet-Off (Clontech) derived cell lines conditionally expressing FLAG-CSN3, FLAG-CUL1 and FLAG-CUL1CΔ31 were established as described previously [29]. To induce the expression of FLAG-tagged proteins, cells were grown without tetracycline for 2 days. Nuclear extracts and cytosolic S100 extracts were prepared as described previously [29]. Extracts were dialyzed against buffer BC (20 mM Tris–HCl [pH 7.9], 15% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, 0.05% NP-40] containing 150 mM KCl (BC150) and rotated with anti-FLAG M2 agarose (Sigma) at 4 °C for 3–6 h. After extensive washes with BC150, proteins were eluted with 0.3 mg FLAG peptide per ml in BC150. Immunopurified protein complexes were resolved on a 4–20%

SDS–polyacrylamide gel (Invitrogen). After gels were stained with colloidal Coomassie brilliant blue G-250, protein bands were excised and digested with trypsin as previously described [35]. In-gel tryptic digests of proteins were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using Voyager-DE STR (Applied Biosystems) and by nanoelectrospray liquid chromatography tandem mass spectrometry (LC–MS–MS) on API QSTAR Pulsar Q-TOF (Applied Biosystems) in the information-dependent acquisition mode. The MS–MS spectra were used to search the National Center for Biotechnology Information (NCBI) nonre-dundant and expressed sequence tag databases using the computer algorithm proID (Applied Biosystems, Framingham, MA).

Immunoprecipitations and Western blotting. Transfection was carried out using the polyethylenenamine methodology [36]. After 36 h, cells were lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride, and 1.0% NP-40. Cell lysates were adjusted to 0.1% NP-40 and incubated with anti-FLAG or anti-HA antibody resin (Sigma) for 4 h at 4 °C. The immune complexes were recovered by low speed centrifugation, and the resin was washed extensively with the binding buffer with 0.1% NP-40 and then eluted with buffer containing 20 mM Tris–HCl (pH 8.0) and 2% SDS or with buffer with 0.3 mg FLAG peptide per ml. Immunoprecipitated proteins were separated by SDS–PAGE and transferred to nitro-cellulose membrane (Bio-Rad) and visualized by Western blotting with the enhanced chemiluminescence reagents (Amersham-Pharmacia). For Western blotting, we purchased commercial antibodies against FLAG (Sigma), HA (Abcam), CAND1, CSN2, CSN5 (BD Biosciences), and CUL1 (Lab Vision).

RNA interference. Specific siRNAs for CAND1 was generated using the Silencer siRNA construction kit (Ambion) according to manufacturer's protocol. Specific siRNAs for GAPDH were provided in the kit. Two 29-mer DNA oligonucleotides for CAND1 (sense, 5′-TGT CTT GGT CCT TTA GTG AGT CCT GTC TC-3′; antisense, 5′-TCA CTA AAG GAC CAA GAC ATT CCT GTC TC-3′) with 21 nucleotides encoding the siRNA for CAND1 and eight nucleotides complementary to the T7 promoter primer were synthesized. The two siRNA templates were hybridized to a T7 promoter primer and were extended by the Klenow DNA polymerase. The extended sense and antisense siRNA templates were then transcribed by T7 RNA polymerase and hybridized to create double-stranded siRNA following the manufacturer's instructions. Twenty picomoles of siRNA were transfected to HeLa cells grown on six-well plates with Lipofect-AMINE 2000 (Life Technologies). Twelve hours later, the expression constructs for FLAG-CUL1 and HA-Rbx1 were transfected into HeLa cells.

Deneddylation assays. Recombinant six-histidine-tagged Nedd8 and Ubc12 were expressed in *Escherichia coli* strain BL21(DE3) and purified using Co^{2+}–agarose beads (Clontech) according to the manufacturer's instructions. The FLAG-Uba3/APP-BP complex was purified from HeLa derived cells stably transfected with an expression vector pYR-FLAG-hUba3 as previously described [29]. Generation of recombinant baculoviruses encoding human CUL1 with a six-His tag and human Rbx1 were previously described [31]. Sf21 cells were co-infected with the recombinant baculoviruses encoding His-CUL1 and Rbx1, and cell lysates were prepared as described [37]. The His-CUL1/Rbx1 complex was purified using Co^{2+}–agarose beads (Clontech) according to the manufacturer's instructions. Recombinant FLAG-His-tagged CAND1 and its deletion mutant were expressed in Sf21 cells [38]. His-CUL1/Rbx1 (1.0 μg) bound to Co^{2+}–agarose beads was neddylated in the reaction mixture containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM NaF, 100 nM okadaic acid, 2 mM ATP, 0.6 mM DTT, 5.0 μg His-Nedd8 , 250 ng FLAG-Uba3/APP-BP, and 1.0 μg Ubc12. Reactions were incubated at 37 °C for 30 min, and after washing the beads, neddylated CUL1 was eluted from the beads as recommended by the manufacturer. Deneddylation reactions were performed in a 30 μl reaction mixture containing 50 mM Tris–HCl (pH