Substrate-mediated Regulation of Cullin Neddylation*

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Cullin-based E3 ligases are a large family of multi-subunit ubiquitin ligases with diverse cellular functions, including the regulation of the cell cycle, of the DNA damage response, and of various transcription factors. These ligases are composed of one of six mammalian cullin homologs (Cul1, Cul2, Cul3, Cul4a, Cul4b, and Cul5), the Ring finger containing protein Roc1/Rbx1, and cullin homolog-specific adaptor and substrate recognition subunits. To be active, cullin-based ligases require the covalent modification of a conserved lysine residue in the cullin protein with the ubiquitin-like protein Nedd8. We show in this study that in intact cells Cul1 neddylation is dependent on binding to adaptor proteins and substrate recognition subunits. Mutant Cul1 that is unable to recruit adaptor and substrate recognition subunits exhibits markedly reduced neddylation, and inhibiting binding of adaptor and substrate recognition subunits to wild type Cul1 reduces Nedd8 modification. This regulatory mechanism also extends to other cullin-based E3 ligases, including Cul2, Cul3, and Cul4a. The regulation of cullin neddylation by adaptor proteins and substrate recognition subunits in cells was found to be independent of both CAND1 and the COP9 signalosome, two negative regulators of cullin Nedd8 modification. Using hypoxia-inducible factor-1α (HIF-1α), a substrate of the Elongin B/C-Cul2-VHL ligase, we demonstrate the critical role of substrate binding to promote Cul2 neddylation in a manner that does not require substrate ubiquitination but may involve a conformational change. These findings suggest a mechanism through which availability of substrate recognition subunits and substrates can regulate the ubiquitin ligase activity.

Cullin-based E3 ligases comprise a large family of ubiquitin ligases that mediate the ubiquitination of numerous cellular proteins with diverse functions including roles in cell signaling, transcriptional regulation, and cell cycle control (1). They are RING domain-containing ligases that function by binding the substrate through a protein-protein interaction domain and the E2 enzyme through the RING motif, thus bringing substrate and E2 enzyme into close proximity and facilitating the transfer of ubiquitin molecules to lysine residues in the substrate. Cullin-based E3 ligases are composed of several subunits, consisting of one of six mammalian cullin homologs (Cul1, Cul2, Cul3, Cul4a, Cul4b, and Cul5) that binds to the RING domain protein Roc1/Rbx1 via its C terminus. The cullin N terminus mediates binding of cullin homolog-specific substrate recognition subunits. Binding of the substrate recognition subunits requires specific adaptor proteins that bridge the interaction with the cullin homologs (except in the case of Cul3). For instance, Cul1 is known to bind substrate recognition subunits containing a conserved F-box via the adaptor protein Skp1, thus forming SCF (Skp1-Cul1-F-box) E3 ligases, whereas Cul2 and Cul5 recruit substrate recognition subunits with a VHL or SOCS box, respectively, via the adaptor proteins Elongin B and C. In contrast, Cul3 is known to bind directly to substrate recognition subunits via their BTB domain (also known as POZ domain). All of the substrate recognition subunits also contain specific protein-protein interaction domains that are responsible for substrate recruitment, often in a manner that is dependent on post-translational modifications of the substrate, such as phosphorylation.

All cullin-based E3 ligases appear to have in common a number of regulatory mechanisms (1). For instance, all cullin-based E3 ligases are covalently modified at a conserved lysine residue at the cullin C terminus with the ubiquitin-like protein Nedd8. Cullin neddylation is required for cullin E3 ligase activity in vivo and is likely to play a role in recruiting E2 ubiquitin-conjugating enzyme to the ligase complex and stimulating multiubiquitin chain assembly (2–4). Neddylation of cullin proteins is mediated by the Nedd8-specific E1 APPBP1-Uba3 heterodimeric enzyme and the E2 enzyme Ubc12 (5, 6). Roc1, the RING domain-containing protein responsible for the E3 ubiquitin ligase activity of cullin-based ligases, is also required for the neddylation of cullins (7).

Another important regulator of cullin-based E3 ligase activity is CAND1, which has been reported to bind to cullin proteins and to specifically interact with the deneddylated form of the protein (8–11). CAND1 binding to cullin proteins disrupts their interaction with adaptor and substrate recognition subunits, thus inhibiting the E3 ubiquitin ligase activity. In addition, the COP9 signalosome (CSN), a complex composed of eight subunits with similarity to the 26 S proteasome lid subcomplex (12–14), is known to deneddylate cullins, which is mediated by the metalloproteinase activity of the CSN5 subunit (15, 16). It has been suggested that cullin proteins undergo cycles of neddylation and deneddylation in vivo (1). However,

* The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; E2, ubiquitin-conjugating enzyme; E1, ubiquitin-activating enzyme; SCF, Skp1-Cul1-F-box; CSN, COP9 signalosome; ECV, Elongin B/C-Cul2-VHL; HIF-1α, hypoxia-inducible factor-1α; HA, hemagglutinin; CDDD, C-terminal oxygen-dependent degradation domain; siRNA, small interfering RNA; DGR, double glycin repeat.

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how cullin neddylation is regulated in cells and coordinated with substrate ubiquitination is currently unknown. Given the requirement of cullin neddylation for the E3 ligase activity in vivo, it is important to identify the mechanisms through which Nedd8 modification of cullins is regulated.

It has been shown that the substrates of the SCF<sup>B-TrCP</sup> E3 ligase, IκBα and β-catenin, associate preferentially with neddylated Cul1 and play a role in promoting the modification of Cul1 with Nedd8 (2, 17). Recently, evidence has been presented for a similar mechanism regulating the neddylation of Cul2 in the Elongin B/C-Cul2-VHL (ECV) E3 ligase complex (18). Furthermore, a recent study reported that binding of the adaptor and substrate recognition subunits Skp1 and Skp2 and of the SCF-Skp<sub>2</sub> substrate p27<sup>Kip1</sup> to Cul1 promotes its neddylation in cell extracts (19). This effect was found to be mediated through Skp1- and Skp2-induced dissociation of CAND1 from Cul1 and substrate-dependent prevention of the action of CSN to deneddylate Cul1 (19). In this report, we extend these studies and show that disrupting the interaction between cullin proteins and their adaptor and substrate recognition subunits in cells reduces neddylation. This dependence on complete E3 ligase assembly for efficient cullin neddylation is a general mechanism that is observed in various cullin-based E3 ligases and appears to be independent of both CAND1 and CSN. Using hypoxia-inducible factor-1α (HIF-1α), a substrate of the ECV E3 ligase, we demonstrate the critical role of substrate binding to promote Cul2 neddylation and provide evidence that this mechanism does not require substrate ubiquitination but is likely to involve a conformational change in the E3 ligase upon binding of the substrate. This is likely to constitute an important regulatory mechanism of cullin-based E3 ligase activity.

**MATERIALS AND METHODS**

**Plasmid Constructs and Mutagenesis**—Skp2 (NM_005983), VHL (NM_000551), and Ubc12 (NM_039969) were cloned from HEK293 cDNA, and human Keap1, murine Cul1 and human Cul2, Cul3, Cul4a, and Cul5 amplified from cDNA Image clones were purchased from Geneservice Ltd. (Image identifiers 3163902, 2812400, 6168545, 3537176, and 30331132, respectively) and inserted into modified pcDNA3.1/Zeo including N-terminal HA-, V5-, or 2×FLAG tags (Ubc12 and Cullin constructs). The dominant-negative cullin constructs dnCul1-V5, and HIF-1α-FLAG according to the manufacturer’s instructions. Mutagenesis was carried out using the Stratagene site-directed mutagenesis kit.

**siRNA-mediated Gene Silencing**—The following annealed Silencer Predesigned siRNA duplexes (Ambion) were used at a final concentration of 50 nM: CAND1: siRNA ID 140585 (CAND1 siRNA1), 27093 (CAND1 siRNA2); CSN5: 214069; negative controls: silencer negative control siRNA 2 and 3 or Mdm2 siRNA 122297. siRNA transfections were carried out using DharmaFECT 1 transfection reagent (Drharmacon) according to the manufacturer’s instructions. The cells were lysed 3 days after siRNA transfections for Western blot analysis.

**Immunoblotting**—For immunoblotting, the cells were washed with ice-cold phosphate-buffered saline and then lysed in Triton X-100-containing lysis buffer, as previously described (22). Lysates were precleared by centrifugation before use for Western blotting. Equal amounts of protein were loaded for Western blot analysis. The following antibodies were used: rabbit polyclonal anti-Cul1 (71–8700; Zymed Laboratories), rabbit polyclonal anti-Cul2 (51–1800; Zymed Laboratories), goat polyclonal anti-CAND1 (A-13) (sc-10672; Santa Cruz Biotechnology), monoclonal anti-p27 (610241; BD Biosciences), goat polyclonal anti-Skp2 (N-19) (sc1567; Santa Cruz Biotechnology), rabbit polyclonal anti-CSN5 (ab12323; Abcam Ltd.), monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (G8140-04; U.S. Biological), monoclonal anti-α-tubulin (236–10501; Molecular Probes), monoclonal anti HIF-1α (610959; BD Biosciences), monoclonal anti-V5 (Serotec), monoclonal anti-FLAG M2 (Sigma), and rat monoclonal anti-HA (clone 3F10) (Roche Applied Science). All of the Western blots shown are representative of three independent experiments.

**Immunoprecipitation**—10 µl of Anti-FLAG M2 agarose (Sigma) or 2.5 µg of V5 antibody, coupled to 15 µl of protein G-Sepharose (Amersham Biosciences), was used for immunoprecipitations. 500 µl of precleared lysate from HEK293 cells transfected in 60-mm tissue culture plates was added. The samples were tumbled at 4 °C for 2 h, and the agarose or Sepharose beads were then washed four times in 1 ml of cold buffer containing 20 mM Tris, pH 7.5, 0.6 M NaCl, and 1 mM EGTA and once in buffer containing 50 mM Tris, pH 7.5. The immunoprecipitated proteins were then denatured in SDS sample buffer and subjected to SDS-PAGE and Western blotting. All of the immunoprecipitation experiments shown are representative of three independent experiments.

**RESULTS**

**Mutant Cullin Proteins That Are Unable to Bind to Adaptor and Substrate Recognition Subunits Exhibit Markedly Reduced Nedd8 Modification**—To characterize the role of substrate recognition subunits and substrates in regulating cullin neddylation, we initially generated Cul1 and Cul3 mutants that based on previous reports are unable to bind to Skp1 and F-box proteins or BTB domain proteins, respectively. Lack of binding of mutant Cul1 Y42A/M43A/E44A (Cul1(YME)) to endogenous and transfected F-box protein Skp2 (Fig. 1, A and B) and of mutant Cul3 S53A/F54A/E55A (Cul3(SFE)) to the BTB domain system (Invitrogen) was used to generate cell lines with tetracycline-inducible expression of dnUbc12-HA (21), dnCul1-V5, dnCul2-V5, and HIF-1α-FLAG according to the manufacturer’s instructions. Mutagenesis was carried out using the Stratagene site-directed mutagenesis kit.
protein Keap1 (Fig. 1C) was confirmed by cotransfection of corresponding expression constructs in HEK293 cells, followed by immunoprecipitation. Skp2 and Keap1 communoprecipitated with wild type but not mutant Cul1 or Cul3, respectively (Fig. 1, A–C). As shown in Fig. 1D, Cul1(YME) and Cul3(SFE), which were unable to bind to their adaptor and substrate recognition subunits, showed only very little Nedd8 modification compared with the wild type proteins, as was apparent by the much lower abundance of the slower migrating band in mutant Cul1 and Cul3. We confirmed that the slower migrating band indeed corresponded to the Nedd8-modified form by coexpressing a dominant-negative version of the Nedd8-conjugating enzyme Ubc12 (C111S) (23), which led to marked reduction of the low mobility band (Fig. 1E).

It can be argued that the primary defect of Cul1(YME) and Cul3(SFE) is the inhibition of Cul1 or Cul3 neddylation and that the absence of binding to Skp2 and Keap1 is a consequence rather than the cause of reduced Nedd8 modification. To rule out this possibility, we examined binding of Skp2 and Keap1 to Cul1 and Cul3, respectively, in a cell line that was stably transinfected with dnUbc12 under control of a tetracycline-inducible promoter (21). Induction of dnUbc12 with tetracycline leads to inhibition of neddylation of cullin proteins, inhibition of their E3 ligase activity, and consequently accumulation of cullin-based E3 ligase substrates, for instance p27Kip1, as shown in Fig. 2(A and B). Inhibition of cullin neddylation had no effect on binding of Skp2 to Cul1 and of Keap1 to Cul3 (Fig. 2, A and B). In addition, mutation of the Cul1 neddylation site Lys720 to Arg prevented Nedd8 modification but did not affect the interaction between Cul1 and Skp2 (Fig. 2C), confirming that cullin neddylation does not regulate binding of substrate recognition subunits.

**Inhibiting Adaptor and Substrate Recognition Subunit Binding to Wild Type Cullin Proteins Reduces Neddylation**—The results in Fig. 1 suggest that Cul1 and Cul3 are only neddylated to a significant degree if they are incorporated into intact E3 ligase complexes. To confirm this hypothesis, we overexpressed...
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FIGURE 3. Effect of dominant-negative cullin expression on neddylation. A, cells were cotransfected with V5-tagged versions of full-length Cul1, Cul2, Cul3, and Cul4a, together with empty vector or the respective dominant-negative cullin (dnCullin) constructs, followed by Western blotting analysis of cell lysates with V5 antibody. B, cells with stable expression of dnCul2-V5 under control of a tetracycline inducible promoter were transfected with Cul2-V5 for 2 days and then treated with 1 μg/ml tetracycline for 18 h, where indicated, followed by Western blotting of cell lysates with V5, HIF-1α, and α-tubulin antibody. The bar graph in the lower panel indicates the ratio of neddylated over unneddylated Cul2-V5. The difference in the ratio in the absence versus presence of tetracycline was statistically significant (n = 3; p < 0.05). C, dnCul2-V5 expression was induced with tetracycline as in B, followed by Western blotting of cell lysates with Cul2, HIF-1α, and glyceraldehyde-3-phosphate dehydrogenase antibody. D, stably transfected cells with tetracycline-inducible expression of dnCul1-V5 were treated with 1 μg/ml tetracycline for 24 h, where indicated, followed by Western blotting of cell lysates using the antibodies shown.

The expression level of transiently transfected dominant-negative Cul2 was low. We therefore generated a HEK293 cell line with stable expression of dnCul2-V5 under control of a tetracycline-inducible promoter. Although there was significant basal expression of dnCul2-V5 in the absence of tetracycline, the addition of tetracycline to the cells induced a marked increase in dnCul2-V5 abundance (Fig. 3B). As expected, induction of dnCul2-V5 resulted in the accumulation of HIF-1α, a substrate of the Cul2-based E3 ligase, but had no effect on α-tubulin expression. The increase in dnCul2-V5 expression upon tetracycline addition was accompanied by a clear reduction in the ratio of neddylated to unneddylated transfected full-length Cul2-V5 (Fig. 3B). A similar reduction in the ratio of Ned8-modified to unmodified Cul2 was also observed for the endogenous protein upon induction of dnCul2 with tetracycline (Fig. 3C). An attempt to perform a similar analysis for Cul5 was technically not possible because the expression of dnCul5-V5 was very low, even in stably transfected clones with tetracycline-inducible expression.

To determine whether endogenous Cul1 is subject to the same regulatory mechanism, a stably transfected HEK293 cell line with tetracycline-inducible expression of dnCul1-V5 was generated (Fig. 3D). There also was significant basal expression of dnCul1-V5 in the absence of tetracycline; however, the addition of tetracycline to the cells for 24 h induced a marked increase in dnCul1-V5 abundance. As expected, this led to the accumulation of the SCFSkp2 substrate p27Kip1, confirming that the dominant-negative, ubiquitination-deficient version of Cul1 can effectively compete with endogenous Cul1 for binding to adaptor, substrate recognition subunits and substrate. As shown in Fig. 3D, dnCul1-V5 induction inhibited neddylation of endogenous Cul1 almost completely.

The Stimulation of Cullin Neddylation by Adapter and Substrate Recognition Subunits Is Not Mediated by CAND1 or CSN—CAND1 and CSN are two important negative regulators of cullin neddylation. CAND1 interacts specifically with unneddylated cullins and binding of CAND1 to Cul1 prevents access to the site of Ned8 modification (11). CSN mediates cullin deneddylation via the metallopeptidase activity of the CSN5 subunit. We therefore considered the possibility that the decreased neddylation of cullin proteins that are not bound to adaptor proteins and substrate recognition subunits is dependent on CAND1 or CSN. To this end, we first examined the binding of CAND1 to the various cullin homologs. V5-tagged cullin homologs were immunoprecipitated from lysates of transfected cells and immunoprecipitates analyzed for coprecipitating CAND1. The results in Fig. 4A show that although all cullin proteins were expressed at similar levels, as apparent from their similar abundance in the V5 immunoprecipitates, only Cul1 bound CAND1 to a significant degree. This result is consistent with previous reports that show that not all cullin proteins interact with CAND1 (9, 24). Although Cul2, Cul3, and Cul4a did not interact with CAND1 in the coimmunoprecipitation assay, binding of adaptor and substrate recognition subunits regulated their neddylation status. We then examined whether the reduced neddylation of the Cul1(YME) mutant that is unable to recruit adaptor and substrate recognition subunits is due to increased CAND1 binding. However, we found that Cul1(YME) showed markedly reduced interaction with CAND1 compared with wild type Cul1 (Fig. 4B). These results indicate that decreased neddylation of binding-deficient cullins is not due to increased association with CAND1. Finally, siRNA-mediated silencing of CAND1 did not increase the neddylation of endogenous Cul1 (Fig. 4C), suggesting that CAND1...
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A, HEK293 cells were transfected with Cul1-V5, Cul2-V5, Cul3-V5, Cul4a-V5, or Cul5-V5, as indicated, followed by immunoprecipitation (IP) of cell lysates with V5 antibody, as described under "Materials and Methods." Immunoprecipitates were then analyzed for coimmunoprecipitating endogenous CAND1 by Western blotting with CAND1 antibody, as indicated, followed by immunoblotting of cell lysates with the indicated antibodies. B, cells were transfected with wild type (wt) Cul1-V5 or Cul1(V5/YME). The cell lysates were subjected to V5 immunoprecipitation and immunoprecipitates analyzed by Western blotting with CAND1 antibody. C, cells were transfected with 50 nm negative control, CAND1 siRNA1 or 2 for 3 days, followed by immunoblotting of cell lysates with the indicated antibodies. D, cells were transfected with 50 nm negative control or CSN5 siRNA for 3 days and cotransfected with expression constructs for Cul1-V5(YME) or Cul3-V5(SFE) for the last 2 days. The cell lysates were used for Western blotting with V5 and CSN5 antibodies.

FIGURE 4. Regulation of cullin neddylation by binding to adaptor and substrate recognition subunits is independent of CAND1 and CSN. A, HEK293 cells were transfected with Cul1-V5, Cul2-V5, Cul3-V5, Cul4a-V5, or Cul5-V5, as indicated, followed by immunoprecipitation (IP) of cell lysates with V5 antibody, as described under "Materials and Methods." Immunoprecipitates were then analyzed for coimmunoprecipitating endogenous CAND1 by Western blotting (WB). B, cells were transfected with wild type (wt) Cul1-V5 or Cul1-V5(YME). The cell lysates were subjected to V5 immunoprecipitation and immunoprecipitates analyzed by Western blotting with CAND1 antibody. C, cells were transfected with 50 nm negative control, CAND1 siRNA1 or 2 for 3 days, followed by immunoblotting of cell lysates with the indicated antibodies. D, cells were transfected with 50 nm negative control or CSN5 siRNA for 3 days and cotransfected with expression constructs for Cul1-V5(YME) or Cul3-V5(SFE) for the last 2 days. The cell lysates were used for Western blotting with V5 and CSN5 antibodies.

FIGURE 5. Requirement of substrate binding for stimulation of Cul1 neddylation by Skp2. The cells were cotransfected with Cul1-HA and full-length (FL) V5-Skp2 or V5-Skp2 (ΔCT), which comprises amino acids 1–249 of Skp2, as indicated. The cell lysates were immunoprecipitated (IP) with V5 antibody, and immunoprecipitates and aliquots of cell lysates were immunoblotted with HA or V5 antibody.

Cul1, presumably by recruiting substrate into the E3 ligase complex.

We then examined neddylation of Cul3 associated with wild type Keap1 and a deletion of Keap1, which lacks the substrate-recruiting double glycine or Kelch repeat (DGR) domain (comprising amino acids 315–598) as well as a short C-terminal region (amino acids 599–624). In contrast to Skp2, wild type Keap1 did not preferentially associate with neddylated Cul3 and the Keap1 mutant deficient in substrate binding immunoprecipitated neddylated and unneddylated Cul3 at the same ratio compared with wild type Keap1 (Fig. 6A). The total amount of Cul3 that coimmunoprecipitated with Keap1(ΔDGR) was greater compared with wild type Keap1, which is likely due to a higher expression level of V5-Keap1(ΔDGR) (Fig. 6A, bottom panel). As shown in Fig. 6B, we confirmed that in contrast to wild type V5-Keap1, V5-Keap1(ΔDGR) is indeed deficient in binding the transcription factor NRF2, the best characterized substrate of Keap1. We thus concluded that the recruitment of substrate into the Keap1-Cul3 ligase complex does not stimulate Cul3 neddylation.

The VHL tumor suppressor protein, which serves as the substrate recognition subunit of the Cul2-based E3 ligase ECV, has been reported to promote Cul2 neddylation (27, 28). Consistent with this, we observed that VHL preferentially associates with neddylated Cul2-FLAG (Fig. 7A). Furthermore, Sufan and Ohh (18) reported that a mutant of VHL that lacks the substrate-binding domain associates exclusively with unneddylated Cul2, demonstrating that substrate binding is required for association of VHL with Neddy8-modified Cul2. Moreover, these authors showed that HIF-1α, the best characterized substrate of the ECV E3 ligase that is recruited through the VHL substrate recognition subunit, associates preferentially with neddylated Cul2 (18). We wished to confirm this result using a cell line with tetracycline-inducible expression of full-length wild type HIF-1α, carrying a C-terminal FLAG tag. As shown in Fig. 7B, significant expression of the HIF-1α-FLAG protein can only be observed in the presence of tetracycline and after incubation of cells under hypoxic conditions. Incubation of cells at low oxygen tension prevents the degradation of the HIF-1α protein by the ECV E3 ligase by inhibiting oxygen-dependent hydroxyla-
Stimulation of Cul2 Neddylation by Substrate Binding Does Not Require Substrate Ubiquitination—To study the role of HIF-1α binding to the ECV complex in regulating Cul2 neddylation further, we used a fusion construct (FLAG-CAT-HIF(546–626) HA) comprised of the HIF-1α CODD (amino acids 546–626, including the Pro564 hydroxylation site) fused to the transactivation domain of β-catenin (amino acids 688–781) and also including an N-terminal FLAG tag (for immunoprecipitation) and a C-terminal HA tag (Fig. 8A). The HIF-1α (amino acids 546–626) is sufficient to interact with VHL upon hydroxylation of Pro564. In contrast to full-length HIF-1α, this fusion protein was well expressed upon transient transfection, and we observed that this was accompanied by increased neddylation of endogenous Cul2 (Fig. 8B). This result suggests that the HIF-1α substrate plays an active role in promoting Cul2 neddylation and does not merely preferentially associate with neddylated Cul2.

To characterize the mechanism through which HIF-1α binding to ECV promotes Cul2 neddylation, we considered the possibility that substrate ubiquitination upon binding to the ligase is involved. To test this, we mutated the two lysine residues in the HIF-1α (amino acids 546–626) domain, Lys547 and Lys625, to Arg (the β-catenin transactivation domain does not contain any lysine residues that could be conjugated with ubiquitin; see also “Discussion”). Wild type and K547R/K625R mutant FLAG-CAT-HIF(546–626)HA were expressed in cells and following incubation of cells in the presence of the proteasome inhibitor MG-132 at a concentration of 25 μM was added for the last 6 h to prevent HIF-1α degradation. The cells were then lysed, and the cell lysates were subjected to immunoprecipitation with FLAG agarose. Aliquots of cell lysates and immunoprecipitates were analyzed using V5 or FLAG antibody. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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| **FIGURE 6. Recruitment of substrate does not regulate the neddylation of Keap1-associated Cul3.** A, HEK293 cells were cotransfected with Cul3-HA and full-length (FL) V5-Keap1 or V5-Keap1(ΔDGR), which comprises amino acids 1–314 of Keap1, as indicated. The cell lysates were immunoprecipitated (IP) with V5 antibody, followed by Western blotting using HA or V5 antibody. B, cells were cotransfected with NRF2-HA and full-length V5-Keap1 or V5-Keap1(ΔDGR), as shown, followed by immunoprecipitation with V5 antibody and Western blotting with HA or V5 antibody. |

| **FIGURE 7. Substrate-mediated stimulation of Cul2 neddylation.** A, HEK293 cells were cotransfected with Cul2-FLAG and V5-VHL, as indicated. The cell lysates were immunoprecipitated (IP) with V5 antibody, followed by Western blotting using FLAG or V5 antibody. B, HEK293 cells stably transfected with HIF-1α-FLAG under a tetracycline-inducible promoter were treated with 1 μg/ml tetracycline for 12 h, where indicated, and then incubated under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions for 6 h. The cell lysates were analyzed with FLAG antibody. C, cells with inducible expression of HIF-1α-FLAG were transfected with Cul2-V5. After 24 h, HIF-1α-FLAG expression was induced with 1 μg/ml tetracycline for 18 h (under normoxic conditions). The proteasome inhibitor MG-132 at a concentration of 25 μM was added for the last 6 h to prevent HIF-1α degradation. The cells were then lysed, and the cell lysates were subjected to immunoprecipitation with FLAG agarose. Aliquots of cell lysates and immunoprecipitates were analyzed using V5 or FLAG antibody. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Cul2 that coimmunoprecipitated with K547R/K625R FLAG-CAT-HIF(546–626)HA was virtually identical compared with wild type protein (Fig. 8C), indicating that substrate ubiquitination is not involved in the stimulation of Cul2 neddylation upon substrate binding. We also noted that the increased neddylation of total endogenous Cul2 neddylation upon expression of FLAG-CAT-HIF(546–626)HA, as observed in Fig. 8B, was not readily detected in the presence of MG-132. Incubation with MG-132 by itself was found to increase Cul2 neddylation (data not shown). Given that we and others have shown that neddylation does not regulate Cul2 stability in mammalian cells (21), we suspect that the increased neddylation of Cul2 upon inhibition of proteasomal protein degradation is due to accumulation of endogenous HIF-1α and other ECV substrates that promote Cul2 neddylation upon binding to VHL or other substrate recognition subunits.

Next, we repeated the experiment in Fig. 8C with cotransfected wild type Cul2-V5 or neddylation site mutant Cul2-V5 (K689R). As shown in Fig. 8D, K689R Cul2 lacked Ned8 modification yet was equally well coimmunoprecipitated compared with wild type Cul2. These results indicate that the preferred association of wild type and K547R/K625R FLAG-CAT-HIF(546–626)HA with neddylated Cul2 is not due to a higher affinity of the substrate for the Ned8-modified Cul2-based E3 ligase. The experiment thus confirms that binding of the HIF-1α substrate to the ECV E3 ligase promotes Cul2 neddylation.

**Mutation of HIF-1α Residues in the VHL Binding Interface Results in Reduced Cul2 Neddylation**—A further possible mechanism for induction of Cul2 neddylation upon binding of the HIF-1α substrate may be a conformational change in the E3 ligase complex. If this was the case, it would be expected that altering the interaction interface between the substrate and the substrate recognition subunit may affect the Cul2 neddylation status. According to the crystal structure of a hydroxylated HIF-1α peptide comprising the CODD (including Hyp564) bound to the Elongin B/C-VHL complex, the HIF-1α substrate interacts with VHL via two binding sites (29, 30). Site 1 (amino acids 561–566, which includes hydroxyproline 564), is the primary binding site and has been shown to be essential for binding of the CODD to VHL, thus precluding mutagenesis of this site. Site 2 (amino acids 571–574) is not required for binding but slightly increases the affinity of the CODD for VHL (29). We mutated Asp571, which is the only residue in site 2 that makes side chain interactions with VHL, and Phe572 to Ala in FLAG-CAT-HIF(546–626)HA and examined binding to Cul2 (Fig. 8E). When compared with wild type FLAG-CAT-HIF(546–626)HA, the D571A/F572A mutant bound less Cul2, consistent with reduced affinity of the mutated CODD to VHL. Although Cul2 that was associated with wild type FLAG-CAT-HIF(546–626)HA was predominantly neddylated, Cul2 in complex with the D571A/F572A mutant was almost completely unneddylated, suggesting that binding of the HIF-1α CODD through both sites 1 and 2 is required to induce Cul2 neddylation.

**DISCUSSION**

Ned8 modification of cullin proteins plays an important role in regulating E3 ligase activity, and it is therefore important to understand the mechanisms through which this post-translational modification is regulated. There is evidence in the literature that the neddylation of cullin proteins is regulated through binding of adaptor proteins, substrate recognition subunits, and substrates. The VHL protein, which serves as a substrate recognition subunit of the ECV Cul2-based E3 ligase, has previously been shown to promote the neddylation of Cul2 (27, 28). Similarly, the F-box protein Cdc4 was found to stimulate Cul1 neddylation in yeast (31). A number of substrate recognition subunits have been observed to preferentially associate with neddylated cullin proteins, including the F-box proteins β-TrCP and Pop1 (2, 17, 32) as well as VHL (Fig. 7A). Finally,
the SCFβ-TrCP E3 ligase substrates IkBα and β-catenin (2, 17) and the ECV E3 ligase substrate HIF-1α (18) were reported to preferentially associate with neddylated Cul1 and Cul2, respectively, and promote cullin neddylation.

In the present study we addressed several outstanding questions regarding the regulation of cullin neddylation by the substrate recognition subunit-substrate complex and provided a number of novel insights. First, we demonstrate that basal neddylation levels observed in vivo are largely dependent on binding of cullin proteins to their substrate recognition subunits. Our results suggest that Ned8-modified cullin proteins present in cells represent cullin proteins that are associated with adaptor and substrate recognition subunits and that presumably form complete E3 ligase complexes. Second, we show that this regulatory mechanism applies to several cullin proteins, including Cul1, Cul2, Cul3, and Cul4A, suggesting that this is a general mechanism through which cullin E3 ligases are regulated.

We furthermore investigated the requirement of substrate binding to the E3 ligase complex for stimulation of cullin neddylation and found that binding of HIF-1α to the ECV E3 ligase induces Cul2 neddylation. These results confirm a recent study by Sufan and Ohh (18). To test whether this requirement for substrate binding is general, we used deletion mutants of Skp2 and Keap1 that lack the substrate-binding domain. We found that the association of Skp2 with neddylated Cul1 requires substrate binding to Skp2. In contrast, the neddylation level of Keap1-associated Cul3 is not regulated through substrate binding to Keap1. On the other hand, we have shown that binding of substrate recognition subunits to Cul3 clearly regulates its neddylation (Figs. 1C and 3A). These results therefore suggest that although the neddylation of all investigated cullin proteins is regulated through binding of substrate recognition subunits, not all substrate recognition subunits appear to be able to induce cullin neddylation in a substrate-dependent manner.

We also investigated putative mechanisms through which the substrate recognition subunit-substrate complex regulates cullin neddylation in intact cells. It has recently been reported that the stimulatory effect of the adaptor and substrate recognition subunits Skp1 and Skp2 and of the substrate p27Kip1 on Cul1 neddylation in HeLa cell extracts is mediated through CAND1 and CSN (19). In contrast, our results argue against a role of CAND1 and CSN in intact cells. Of note, Rub1/Nedd8 modification of Cdc53, the Cul1 ortholog in budding yeast, which lacks an obvious CAND1 homolog and all CSN subunits except for CSN5, is also dependent on Skp1 and stimulated by the F-box protein Cdc4 (31), suggesting that CAND1- and CSN-independent regulation of cullin neddylation by binding of adaptor proteins, substrate recognition subunits and substrates is a conserved mechanism.

We subsequently explored alternative mechanisms for the regulation of cullin neddylation by the substrate recognition subunit-substrate complex. Given the diversity of cullin-based E3 ligase substrate recognition subunits and substrates, but their apparently related effects on cullin neddylation, we considered the possibility that the stimulation of cullin neddylation is dependent on substrate ubiquitination, which is common to all E3 ligase substrates. To study the role of substrate ubiquitination, we used a fusion protein that comprises the CODD of HIF-1α, a substrate of the ECV ubiquitin ligase, fused in frame to the β-catenin transactivation domain (FLAG-CAT-HIF(546–626)HA). A mutant that lacked any lysine residues in both the HIF-1α CODD and the β-catenin transactivation domain was found to still preferentially associate with neddylated Cul2, suggesting that conjugation of ubiquitin onto lysine residues in the substrate is not required for the stimulation of cullin neddylation. One potential caveat is the presence of a number of lysine residues in the N-terminal FLAG tag of the FLAG-CAT-HIF(546–626)HA through which ubiquitination may occur. (Alternatively, it is also conceivable that ubiquitination occurs through the N-terminal free amino group of the fusion protein.) However, given that the E3 ligase-binding site and the potential ubiquitination sites in FLAG-CAT-HIF(546–626)HA are separated by ~115 amino acids in the K547R/K625 mutant, compared with a distance of 17 amino acids to the nearest lysine residue in the wild type fusion protein, one would expect that ubiquitination would at least be markedly reduced in the mutant. However, we observed identical effects of wild type and K547R/K625 mutant FLAG-CAT-HIF(546–626)HA. Moreover, ubiquitination of lysine residues at the N terminus is highly expected to mask the FLAG epitope that was used to immunoprecipitate the fusion proteins from cell lysate. However, we observed that the wild type and K547R/K625 mutant proteins were equally well immunoprecipitated (Fig. 8, C and D), supporting the conclusion that substrate ubiquitination is not involved in the regulation of cullin neddylation.

Our results suggest that binding of the substrate may be sufficient to promote cullin neddylation, possibly by inducing a conformational change in the E3 ligase complex. In support of this hypothesis, we found that the HIF-1α CODD with two mutated residues in the VHL binding site 2 still bound to VHL (although with reduced affinity) but was unable to induce the neddylation of Cul2. These results suggest that binding of the HIF-1α CODD to VHL by itself is not sufficient but that correct association of the CODD with VHL that includes the binding site 2 in the HIF-1α peptide is required. We propose that this may lead to a conformational change in the E3 ubiquitin ligase complex that results in the stimulation of the Nedd8 modification of the Cul2 protein. In support, some rearrangements of the three subunits of the VHL-Elongin B/C complex that are distant from the CODD-binding site were noted in the crystal structures of the substrate-bound compared with the unbound complex (30). It is possible that such a conformational change results in increased accessibility of the conserved lysine residue in the cullin protein that is the target of Nedd8 conjugation. However, given the distance between the substrate recognition subunit-binding site and the neddylation site in the cullin protein, we would favor a model in which substrate binding induces a change in the composition of the ligase complex, for instance through dimerization of the ligase complex or a change in the affinity for binding proteins that mediate or regulate cullin Nedd8 modification. Further biochemical and structural studies will be required to identify the molecular mechanism through which substrate binding leads to cullin neddylation.
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REFERENCES

1. Petroski, M. D., and Deshaies, R. J. (2005) Nat. Rev. Mol. Cell Biol. 6, 9–20
2. Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimada, N., Hidaka, Y., Osaka, F., Omata, M., and Tanaka, K. (2001) EMBO J. 20, 4003–4012
3. Wu, K., Chen, A., Tan, P., and Pan, Z. Q. (2002) J. Biol. Chem. 277, 167–168
4. Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998) EMBO J. 17, 2208–2214
5. Gong, L., and Yeh, E. T. (1999) J. Biol. Chem. 274, 12036–12042
6. Kamura, T., Conrad, M. N., Yan, Q., Conaway, R. C., and Conaway, J. W. (1999) Genes Dev. 13, 2928–2933
7. Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (2002) Mol. Cell 10, 1511–1518
8. Min, K. W., Hwang, J. W., Lee, J. S., Park, Y., Tamura, T. A., and Yoon J. B. (2003) J. Biol. Chem. 278, 15905–15910
9. Goldenberg, S. I., Cascio, T. C., Shumway, S. D., Garbutt, K. C., Liu, J., Xiong, Y., and Zheng, N. (2004) Cell 119, 517–528
10. Wolf, D. A., Zhou, C., and Wee, S. (2003) Nat. Cell Biol. 5, 1029–1033
11. Wei, N., and Deng, X. W. (2003) Annu. Rev. Cell Dev. Biol. 19, 261–286
12. Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D. A., Wei, N., Shevchenko, A., and Deshaies, R. J. (2001) Science 292, 1382–1385
13. Cope, G. A., Suh, G. S., Aravind, L., Schwarz, S. E., Zipursky, S. L., Koonin, E. V., and Deshaies R. J. (2002) Science 98, 608–611
14. Read, M. A., Brownell, J. E., Gladyshova, T. B., Hottelet, M., Parent, L. A., Coggins, M. B., Pierce, J. W., Podust, V. N., Luo, R. S., Chau, V., and Palombella, V. J. (2000) Mol. Cell. Biol. 20, 2326–2333
15. Sufan, R. I., and Oeh, M. (2006) Neoplasia 8, 956–963
16. Bornstein, G., Ganoth, D., and Hershko, A. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 11515–11520
17. Hagen, T., Taylor, C. T., Lam, F., and Moncada, S. (2003) Science 302, 1975–1978
18. Culbert, A. A., Brown, M. J., Frame, S. M., Hagen, T., Cross, D. A. E., Bax, B., and Reith, A. D. (2001) FEBS Lett. 507, 288–294
19. Wada, H., Yeh, E. T. H., and Kamitani, T. (2000) J. Biol. Chem. 275, 17008–17015
20. Oshikawa, K., Matsumoto, M., Yada, M., Kamura, T., Hatakeyama, S., and Nakayama, K. I. (2003) Biochem. Biophys. Res. Commun. 303, 1209–1216
21. Schulman, B. A., Carrano, A. C., Jeffrey, P. D., Bowen, Z., Finnin, M. S., Elledge, S. J., Harper, J. W., Pagano, M., and Pavletich, N. P. (2000) Nature 408, 381–386
22. Hao, B., Zheng, N., Schulman, B. A., Wu, G., Miller, J. J., Pagano, M., and Pavletich, N. P. (2005) Mol. Cell. Biol. 20, 9–19
23. Wada, H., Yeh, E. T. H., and Kamitani, T. (1999) J. Biol. Chem. 274, 36025–36029
24. Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., Callis, J., Goebl, M., and Estelle, M. (1998) Genes Dev. 12, 914–926
25. Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh-e, A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2000) EMBO J. 19, 3475–3484