The COP9/Signalosome Increases the Efficiency of von Hippel-Lindau Protein Ubiquitin Ligase-mediated Hypoxia-inducible Factor-α Ubiquitination

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Oxygen-dependent ubiquitination of the α-subunit of hypoxia-inducible factor (HIF-α) by the (von Hippel-Lindau protein)-Elongin B/C-Cullin2-Rbx1 (VBC-Cul2) ubiquitin ligase, a member of the cullin-RING ubiquitin ligases (CRLs), plays a central role in controlling oxygen metabolism. Nedd8 conjugation of cullins enhances the ligase activity of CRLs, and the COP9/signalosome (CSN) enhances the degradation of several CRL substrates, although it removes Nedd8 from cullins. Here we demonstrate that CSN increased the efficiency of the VBC-Cul2 complex for recognizing and ubiquitinating substrates by facilitating the dissociation of ubiquitinated substrates from the pVHL subunit of the complex. Moreover CSN enhanced HIF-1α degradation by promoting the dissociation of HIF-1α from pVHL in cells. The length of the polyubiquitin chain conjugated to the substrate appeared to be involved in CSN-mediated dissociation of the substrate from pVHL. In contrast to other mechanisms underlying CSN-mediated activation of CRLs, the dissociation of ubiquitinated substrates from pVHL did not require the deneddylation activity of CSN, implying that CSN enhances degradation of CRL substrates by multiple mechanisms.

Hyoxia-inducible factors (HIFs) are the central regulators of mammalian oxygen metabolism because of their ability to enhance the expression of hypoxia-inducible mRNAs in hypoxic conditions. HIFs are heterodimers consisting of a labile α-subunit (HIF-α) and a β-subunit (HIF-β). The oxygen-dependent degradation of HIF-α (HIF-1α and HIF-2α) plays a central role in the regulation of oxygen metabolism (1). In oxygenated cells, specific proline residues in the oxygen-dependent degradation (ODD) domain of HIF-α (Pro-402 and Pro-564 in HIF-1α and Pro-405 and Pro-531 in HIF-2α) are hydroxylated by a member of the EglN family of 2-oxoglutarate-dependent dioxygenases (2, 3). Hydroxyproline residues in the HIF-α ODD are specifically recognized by pVHL, the substrate recognition subunit of the pVHL-Elongin B/C-Cullin2-Rbx1 (VBC-Cul2) ubiquitin ligase (4), resulting in ubiquitin-mediated degradation of HIF-α (5, 6). pVHL, the gene product of VHL, lead to susceptibility to von Hippel-Lindau disease, a condition characterized by a variety of tumors. Additionally pVHL is a tumor suppressor of sporadic clear cell renal carcinomas (7). Thus, pVHL-mediated HIF-α degradation is involved in tumor progression as well as oxygen sensing (8).

VBC-Cul2 is a member of the cullin-RING ubiquitin ligase (CRL) family of which Skp1-cullin1-F box protein (SCF) is the prototype (9). The ubiquitin ligase activity of CRLs, including VBC-Cul2, is enhanced by conjugation of a ubiquitin-like protein, Nedd8, to the cullin (neddylation) (9, 10). The COP9/signalosome (CSN), which is composed of eight subunits (CSN1-8), removes Nedd8 from cullins (deneddylation) (11, 12), but at the same time, CSN activates the degradation of substrates of the Cul1 and Cul3-based CRLs in vivo (12–14). However, the role CSN plays in degradation of HIF-α by VBC-Cul2 has not been well studied. We therefore examined the effect of CSN on HIF-α ubiquitination in an in vitro ubiquitination assay and identified a novel mechanism underlying CSN-mediated activation of CRLs.

EXPERIMENTAL PROCEDURES

Generation of Plasmids, Recombinant Baculoviruses, and Recombinant Proteins—Open reading frames of human CSN5 and CSN1 were amplified by RT-PCR from HeLa cell mRNA. cDNAs for Nedd8, Rbx1, pVHL, and the ODD domain of HIF-2α have been described previously (15). Mutants were constructed by two-step PCR. Open reading frames linked to appropriate tags were cloned into pcDNA3.1, pVL1393 (Invitrogen), or pMal-c2x (New England Biolabs). A DNA fragment encoding the N-terminal 54 amino acids of IxBα (IxBα(1–54)) was cloned into the pGEX-6p-1 vector (GE Healthcare). Recombinant baculoviruses encoding His6-Rbx1 and His6-pVHL-HA were generated using the Bac-Pak6 bacu-
lovirus expression system (Clontech). Recombinant baculoviruses for Myc-Cul2, Elongin B, Elongin C, FLAG-pVHL, T7-Rbx1, His6-APP-BP1, T7-Uba3, Myc-Cul1, His6-βTrCP1, FLAG-Skp1, His6-IKKβ-EE (Ser-177 and -181 are replaced by Glu), and His6-E1 have been described previously (4, 16). Expression and purification of recombinant proteins in High Five insect cells or in bacterial cells was performed as described previously (15, 17). For purification of E3s containing multiple components, High Five cells were co-infected with recombinant baculoviruses expressing each component of the ligase complex (Elongin B, Elongin C, and His6-pVHL-HA for VBC; Myc-Cul2 and His6-Rbx1 for Cul2-Rbx1; Elongin B, Elongin C, His6-pVHL-HA, Myc-Cul2, and T7-Rbx1 for VBC-Cul2; Myc-Cul1 and T7-Rbx1 for Cul1-Rbx1; and FLAG-Skp1 and His6-βTrCP1 for Skp1-βTrCP1) followed by purification as described previously (15).

**Antibodies**—Anti-HIF-1α and anti-HIF-2α were purchased from Novus Biologicals. Anti-Myc and anti-HA were purchased from Covance. Anti-maltose-binding protein (MBP) and anti-glutathione S-transferase were obtained from Santa Cruz Biotechnology. Anti-FLAG M2, anti-His6, and anti-Myc antibodies were added to the purification mixtures and incubated on ice for 2 h followed by removal of antibody-reactive materials with protein A-Sepharose (GE Healthcare). The unabsorbed materials were concentrated by trichloroacetic acid precipitation.

**Immunoprecipitation and Immunoblotting**—Cells were lysed in ice-cold buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, 1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride followed by centrifugation at 15,000 × g for 20 min at 4 °C. For immunoprecipitation, cell lysates were incubated with the appropriate antibodies on ice for 2 h followed by precipitation with protein A-Sepharose. For immunoblotting, samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membrane was incubated with the appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), and antibody binding was visualized using the SuperSignal chemiluminescence detection system (Pierce). Detection and quantitation were performed using a LAS3000 image analyzer (Fuji Film).

**Purification of the CSN Complex**—To purify CSN wild type (CSN-WT) or mutant CSN containing CSN5 with Asn substituted for Asp-151 (CSN-D151N), U2OS cells expressing FLAG-CSN1 and His6-CSN5 or His6-CSN5-D151N were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride using a Dounce homogenizer followed by centrifugation at 100,000 × g for 1 h. The lysates were incubated with nickel-nitrotriacetic acid resin (Qiagen) followed by elution with 100 mM imidazole. The eluate was then incubated with anti-FLAG M2 beads (Sigma), and the bound material eluted with FLAG peptide (Sigma).

**In Vitro Ubiquitination Assays**—The conjugation mixture contained (in a final volume of 20 μl) 60 nM prolyl-hydroxylated HIF-α ODD domain fused to MBP (MBP-ODD), 40 nM E1, 150 nM E2 (UbcH5c), 20 nM Tris-HCl (pH 7.5), 5 mM MgCl2, 2 mM dithiothreitol, 2 mM 2-oxoglutarate, 2 mM ascorbic acid, 1 mM EGLN3, 30 μM ubiquitin, 200 nM Cul2-Rbx1, and 200 nM VBC containing FLAG-pVHL in the presence of the neddylation system unless otherwise indicated. The neddylation system consists of 20 nM APP-BP1-Uba3, 500 nM Ubc12, and 6 μM Nedd8. In some experiments, 6 μM ubiquitin aldehyde (Ubal) (Boston Biochem) was added. The reaction mixtures were incubated in the presence of ATP and an ATP-regenerating system (0.5 mM ATP, 10 mM creatine phosphate, and 100 μM creatine phosphokinase) for 60 min at 37 °C. Any differences from the assay components described above are indicated in the figure legends. The **in vitro** ubiquitination assay of GST-κBo-(1–54) by SCFβTrCP1 was carried out as described previously (17).

**RESULTS**

**CSN Facilitates the Ubiquitination of Substrates by VBC-Cul2 Ligase**—We first probed the effect of neddylation on substrate ubiquitination by the VBC-Cul2 ligase in our **in vitro** ubiquitination assay using purified proteins by measuring VBC-Cul2-mediated ubiquitination of the prolyl-hydroxylated ODD domain of HIF-2α fused to maltose-binding protein (MBP-ODD) (Ref. 15 and supplemental Fig. 1, A and B). The Nedd8 conjugation (neddylation) system, which is composed of the APP-BP1-Uba3 complex, Ubc12, and Nedd8, increased the amount of ubiquitin moieties that were conjugated to the MBP-ODD after 60 min incubation (Fig. 1A). To examine the effect of the neddylation system more precisely, MBP-ODD was incu-
bated as described for Fig. 1A, and the reaction was stopped at several time points before 60 min (Fig. 1B). The number of ubiquitin moieties conjugated to MBP-ODD was higher in the presence of the neddylation system than in its absence at all the time points examined, including as early as 15 min.

Although CSN enhances substrate degradation in vivo (12–14), the complex removes Ned8 from cullins in vitro (11, 12). This seems to contradict the observation that neddylation enhances substrate ubiquitination by CRLs, including VBC-Cul2 (10), and increases the number of ubiquitins conjugated to MBP-ODD by VBC-Cul2. We therefore probed the effect of CSN on MBP-ODD ubiquitination using an in vitro ubiquitination assay with purified components. The CSN complex was purified from U2OS cells expressing His6-CSN5 and FLAG-CSN1 (supplemental Fig. 2A). First we assessed the effect of CSN on MBP-ODD ubiquitination by adding the indicated concentration of CSN and found that the amount of ubiquitin-conjugated MBP-ODD increased in the presence of as little as 30 nM CSN (Fig. 2A, note the decrease in unconjugated MBP-ODD in lanes 6–9 compared with lane 4). Because CSN possesses deneddylation activity, we next probed the effect of Cul2 neddylation on the CSN-mediated increase of ubiquitinated MBP-ODD. MBP-ODD was incubated in the presence or absence of the neddylation system for 60 min (Fig. 2B). We confirmed that the neddylation system increases the number of ubiquitin moieties that were conjugated to MBP-ODD (compare the increase in the laddered signals in lanes 7 and 6 with lanes 5 and 4, respectively). CSN decreased the amount of

FIGURE 1. The Nedd8 conjugation system increases the number of ubiquitinated MBP-ODD molecules. A, VBC-Cul2 conjugates more ubiquitin moieties (\((Ub)n\)) to MBP-ODD in the presence of the neddylation system. MBP-ODD was incubated with the indicated components for 60 min as described under "Experimental Procedures." Unmodified MBP-ODD is indicated at the bottom of the gel; bands of higher molecular mass indicate increasing ubiquitination. B, the neddylation system enhanced VBC-Cul2-mediated ubiquitination of MBP-ODD. MBP-ODD was incubated in the presence or absence of the neddylation system for the indicated times. Following incubation with the indicated components, samples were separated by SDS-PAGE followed by immunoblotting with anti-MBP (A and B) or anti-Myc to label Myc-Cul2 (B).

FIGURE 2. The CSN complex increases the number of ubiquitinated MBP-ODD molecules. Following incubation with the indicated components, samples were separated by SDS-PAGE followed by immunoblotting with anti-MBP. A, CSN increases the amount of ubiquitinated MBP-ODD. MBP-ODD was incubated with the indicated concentrations of CSN and 300 nM UbcH5c in the presence or absence of the neddylation system or ubiquitin. B, the neddylation system appears not to be involved in the CSN-mediated increase in the amount of ubiquitinated MBP-ODD. C, CSN increased the amount of the ubiquitinated MBP-ODD at all time points examined except 15 min. MBP-ODD was incubated with 300 nM UbcH5c in the presence or absence of CSN for the indicated time. D, increasing amounts of UbcH5c counteract the CSN-mediated increase in the number of ubiquitins conjugated to MBP-ODD. MBP-ODD was incubated with the indicated amounts of CSN and UbcH5c. E, the CSN-mediated increase in the amount of ubiquitinated MBP-ODD seems to be correlated with the number of ubiquitin moieties conjugated to MBP-ODD. MBP-ODD was incubated as depicted for the indicated periods in the presence or absence of Ubal. F, relative amounts of unmodified MBP-ODD in E. Ub, ubiquitin.
unconjugated MBP-ODD regardless of the presence of the neddylation system (compare lanes 7 and 5 with lanes 6 and 4, respectively), although the CSN-mediated increase in the amount of ubiquitinated MBP-ODD was smaller in the absence than in the presence of the neddylation system. To examine the effect of CSN on MBP-ODD ubiquitination kinetically, we terminated the in vitro ubiquitination reactions at several time points (Fig. 2C). The number of ubiquitin moieties conjugated to MBP-ODD increased in proportion to the incubation period. At the same time, the amount of unconjugated MBP-ODD became smaller in the presence of CSN at 30, 45, and 60 min than in the absence of CSN, suggesting that CSN facilitates the ubiquitination of MBP-ODD by the ligase. However, CSN did not decrease the amount of unconjugated MBP-ODD when the reaction was terminated at 15 min. CSN associates with the deubiquitination enzyme USP15, and we have shown that USP15 associates with the purified CSN complex used in our assay (supplemental Fig. 2C and Refs. 18 and 19). Moreover we observed that MBP-ODD ubiquitination was suppressed in the presence of a higher concentration (1 μM) of CSN (Fig. 2A, compare lane 3 with lane 5), and MBP-ODD ubiquitination was more profoundly suppressed in the absence of the neddylation system (Fig. 2, A, compare lane 5 with lane 6, and B, compare lane 4 with lane 5). We therefore suspected that CSN counteracts substrate ubiquitination by CRLs through its interactions with USP15. To examine whether the deubiquitination activity is indeed associated with the purified CSN complex we used here, we incubated MBP-ODD with different amounts of UbcH5c and CSN (Fig. 2D). The number of ubiquitin moieties conjugated to MBP-ODD was decreased in the presence of the larger amount of CSN (compare unconjugated MBP-ODD in lanes 1, 5, and 9), but the increased amount of E2 (UbcH5c) counteracted the decrease of the ubiquitinated MBP-ODD (compare lanes 9–12). This indicates that the deubiquitination activity is indeed associated with the purified CSN complex and may prevent us from evaluating the amount of unconjugated MBP-ODD in our assay system. To address this, we added Ubal, an inhibitor of debiquitinating enzymes, to the in vitro ubiquitination assay to inhibit the debiquitination activity associated with CSN and terminated the reaction at the indicated time points (Fig. 2, E and F). Ubal substantially, although not completely, inhibited the debiquitination activity associated with CSN (Fig. 2E, compare the laddered signals in lane 7 with lane 13). CSN reduced the amount of unmodified MBP-ODD in the presence of Ubal at all time points examined, including as early as 10 min (Fig. 2E). Moreover the CSN-mediated decrease in the amount of unmodified MBP-ODD was enhanced with longer incubation (Fig. 2, E and F). Because the amount of the E3 added to the in vitro reaction mixture was identical in all the assays, these results strongly indicated that CSN facilitates the ubiquitination of substrates by a single VBC-Cul2 complex, and longer polyubiquitin chains might increase the effect mediated by CSN.

The Increase in Efficiency of VBC-Cul2-mediated Substrate Ubiquitination by CSN Does Not Require the CSN Deneddylation Activity—HIF-α is rapidly degraded by the 26 S proteasome after ubiquitination by VBC-Cul2 (5, 6). To be recognized by the 26 S proteasome, HIF-α must be conjugated with polyubiquitin chains but not subjected to mult ubiquitination (monoubiquitination at multiple sites; Ref. 20). Because there are four Lys residues in the ODD domain of HIF-2α (Lys-429, Lys-497, Lys-503, and Lys-512; the numbering represents Lys residues in HIF-2α), CSN may facilitate the conjugation of multiple polyubiquitin chains to MBP-ODD, which could increase the amount of ubiquitinated MBP-ODD. If this occurred, the length of each polyubiquitin chain conjugated to a substrate might be too short to be recognized by the 26 S proteasome for degradation (20) because we observed that CSN does not increase the amount of ubiquitin conjugated to MBP-ODD (Fig. 2E, compare lanes 10 and 12 with lanes 11 and 13, respectively). To test this, we generated MBP-ODD with no Lys (MBP-ODD-K0) or one Lys (MBP-ODD-Lys-429, -Lys-497, -Lys-503, or -Lys-512) by substituting Arg for Lys at the appropriate positions and assessed the effect of CSN on the ubiquitination of these MBP-ODD mutants (Fig. 3A). MBP-ODD-K0 and MBP-ODD-Lys-429 were only weakly ubiquitinated by VBC-Cul2. However, MBP-ODD-Lys-497, -Lys-503, and -Lys-512 were polyubiquitinated, although ubiquitination of these mutants was weaker than that of MBP-ODD, indicating that VBC-Cul2 conjugates polyubiquitin chains to these Lys residues in the HIF-2α ODD, not to any Lys in MBP. Incubation of MBP-ODD-Lys-497, -Lys-503, and -Lys-512 with 300 nM UbcH5c in the presence of CSN for 60 min increased the amounts of ubiquitinated MBP-ODD mutants (Fig. 3B).

We next examined the effect of CSN on ubiquitination of MBP-ODD-Lys-503 kinetically by terminating the reaction at several time points (Fig. 3C). The length of the polyubiquitin chain was proportional to the incubation period, and CSN decreased the amount of unmodified MBP-ODD-Lys-503 after incubations of at least 30 min. However, CSN did not decrease the amount of unconjugated MBP-ODD-Lys-503 after a 15-min incubation. We then added Ubal to the in vitro ubiquitination assays to inhibit the deubiquitination activity associated with the CSN complex (Fig. 3, D and E). Ubal facilitated the CSN-mediated increase in the amount of ubiquitinated MBP-ODD-Lys-503 as we had observed with wild type MBP-ODD (Fig. 2E). The CSN-mediated decrease in the amount of unmodified MBP-ODD was observed at all time points tested and was enhanced when the substrate was incubated for a longer period. Moreover the enhancement of the CSN-mediated decrease in the amount of unmodified MBP-ODD seemed to correlate with the length of the polyubiquitin chain conjugated to MBP-ODD-Lys-503 (see laddered signals in Fig. 3, D and E). These results strongly indicate that CSN stimulates VBC-Cul2-mediated ubiquitination of MBP-ODD by increasing the number of MBP-ODD ubiquitinated by a single E3 complex, and the length of the conjugated polyubiquitin chain is critical for the effect mediated by CSN.

Because deneddylation is the only known enzyme activity of the CSN complex, we examined whether the deneddylation activity of CSN is involved in the increase in efficiency of VBC-Cul2-mediated MBP-ODD ubiquitination. The CSN5 subunit of CSN is the catalytic subunit, and substitution of Asp-151 in CSN5 with Asn (CSN5-D151N) abolishes the deneddylation activity (12). The mutant CSN complex (CSN-D151N) purified from U2OS cells expressing His6-CSN5-D151N and FLAG-
CSN1 failed to deneddylate Cul2 (supplemental Fig. 2, A and B). To examine whether the deneddylation activity of CSN is involved in CSN-mediated enhancement of ubiquitinated substrates, we incubated MBP-ODD with either the CSN-WT or CSN-D151N in the presence or absence of the neddylation system for 60 min (Fig. 3F). The deneddylation-defective CSN complex decreased the amount of unconjugated MBP-ODD as effectively as CSN-WT regardless of the presence of the neddylation system (Fig. 3F). However, we examined the effect of CNS-D151N only after a 60-min incubation and realized that, at this time, the reaction might almost reach the end point because, as shown in Fig. 3C, the length of the polyubiquitin chain as well as the amount of unconjugated MBP-ODD remained in the presence of CSN after the 60-min incubation, suggesting that MBP-ODD is in excess in our assay conditions. In that situation, CSN-D151N could decrease the amount of unconjugated MBP-ODD as much as CSN-WT (Fig. 3F). Thus, the result indicated that the deneddylation activity of CSN is not essential for the effect, although we could not rule out completely the possibility that the deneddylation activity may play a limited role in CSN-mediated increase of ubiquitinate MBP-ODD. Collectively these results indicate that CSN enhances the ubiquitin ligase activity of the VBC-Cul2 ligase by increasing the efficiency by which a ligase complex recognizes and ubiquitinates prolyl-hydroxylated HIF-α, and the deneddylation activity of CSN does not appear to be critical for this activation, although we cannot rule out completely the possibility that deneddylation is involved.

**CSN Induces the Dissociation of MBP-ODD from VBC-Cul2**—We next examined how CSN increases the efficiency of MBP-ODD ubiquitination by the VBC-Cul2 ligase. CSN leads to the dissociation of the Skp1-F box complex from Cul1 by removing Nedd8 from Cul1 (9). VBC-Cul2 can also dissociate into VBC and Cul2-Rbx1 (15). Thus, CSN may augment the dissociation of VBC together with bound ubiquitinated substrates from Cul2-Rbx1 and facilitate association of pVHL recognizing unmodified substrates to Cul2-Rbx1, which would enhance the efficiency of the ubiquitination because the Cul2-Rbx1 complex is essential for the transfer of ubiquitin from an E2 to a substrate, such as MBP-ODD. If this were the case, a molar excess of VBC relative to Cul2-Rbx1 might suppress MBP-ODD ubiquitination in the absence of CSN, and CSN might counteract this suppression. We added VBC and Cul2-Rbx1 separately to the *in vitro* ubiquitination assay mix-
ever, in the absence of CSN, 300 or 500 nM VBC increased the presence of any amount of VBC tested (Fig. 4A). To confirm this, we next used 200 nM VBC-Cul2-Rbx1 complex was purified from insect cells co-infected with recombinant baculoviruses expressing each component of the complex. In the absence of CSN, the addition of either 20 or 60 nM free VBC (1/10 and 3/10 the amount of Cul2-Rbx1, respectively) increased the amount of unmodified MBP-ODD when incubated for 60 min (Fig. 4B). However, in the presence of CSN, 20 nM free VBC did not increase the amount of unmodified MBP-ODD (compare lane 2 with lanes 4 and 6). Although 60 nM free VBC slightly increased the amount of unmodified MBP-ODD (compare lane 7 with lane 6), the amount of unmodified MBP-ODD in the sample was still less than that in the sample without CSN and free VBC (compare lane 7 to lane 2). Basically the same results were obtained when the reactions were terminated at 30 min (supplemental Fig. 3). Although the effect of excess VBC is more potent in mixtures containing VBC and Cul2-Rbx1 (Fig. 4A) relative to those containing VBC-Cul2-Rbx1 (Fig. 4B) these results clearly indicate that CSN counteracts the decrease in the amount of ubiquitinated MBP-ODD mediated by free VBC. This supports our hypothesis that CSN facilitates the dissociation of VBC and ubiquitinated MBP-ODD from Cul2-Rbx1 and induces the association of free VBC with unmodified MBP-ODD.

If this hypothesis is correct, substrates ubiquitinated by the ligase complex must dissociate from Cul2-Rbx1. Thus, the amount of ubiquitinated MBP-ODD that is not associated with Cul2 in the in vitro reaction mixtures must be larger in the presence than in the absence of CSN. To test this, we immunoprecipitated Myc-Cul2 from the in vitro ubiquitination reaction mixtures with a saturating amount of anti-Myc antibody, incubated the reactions in the presence or absence of the indicated amount of CSN for 60 min, and tried to assess the amount of unmodified MBP-ODD mediated by free VBC. This was done by immunoabsorbing material from Cul2 with an equal amount of preabsorption material and an equal amount of preabsorption material and an equal amount of preabsorption material (Fig. 4C). The VBC-Cul2-Rbx1 complex was purified by immunoblotting with anti-Myc to remove Myc-Cul2. Samples (15%) of Cul2-immunoabsorbed (Cul2 absorbed) material were separated by SDS-PAGE followed by immunoblotting with anti-MBP and anti-HA. C, CSN facilitates dissociation of ubiquitinated MBP-ODD and VBC from Cul2. MBP-ODD was ubiquitinated with 600 nM UbcH5c and VBC containing HA-pVHL in the presence or absence of the induced concentration of CSN followed by immunoblotting with an anti-Myc antibody to remove Myc-Cul2. Samples (15%) of Cul2-immunoabsorbed (Cul2 absorbed) material were separated by SDS-PAGE together with an equal amount of preabsorption material (Input) followed by immunoblotting with anti-MBP and anti-HA. D, CSN facilitates dissociation of ubiquitinated MBP-ODD from pVHL. MBP-ODD was ubiquitinated as in C followed by immunoblotting with pVHL-immunoabsorbed (pVHL absorbed) material and an equal amount of preabsorption material (Input) were separated by SDS-PAGE followed by immunoblotting with anti-MBP. E, deubiquitylation-defective CSN facilitates dissociation of ubiquitinated MBP-ODD and pVHL from Cul2. MBP-ODD was ubiquitinated as in C in the presence or absence of CSN containing CSN5-D151N followed by immunoblotting with anti-Myc to remove Myc-Cul2. Samples (15%) of Cul2-immunoabsorbed material and an equal amount of preabsorption material (Input) were separated by SDS-PAGE followed by immunoblotting as in C. F, CSN containing CSN5-D151N also facilitates dissociation of ubiquitinated MBP-ODD from pVHL. MBP-ODD was incubated as in E followed by immunoblotting of pVHL and immunoblotting as in D. Ub, ubiquitin.

FIGURE 4. CSN induces the dissociation of MBP-ODD from the ligase complex. A, excess VBC decreased ubiquitinated MBP-ODD in the absence of CSN. MBP-ODD was incubated in the absence of VBC with 300 nM UbcH5c. Samples were separated by SDSPAGE and immunoblotted with anti-MBP. B, addition of free VBC decreased ubiquitinated MBP-ODD in the absence of CSN. MBP-ODD was incubated with the VBC-Cul2-Rbx1 complex in the presence or absence of the indicated amount of free VBC and 300 nM UbcH5c for 60 min. Samples were separated by SDSPAGE and immunoblotted with anti-MBP. C, CSN facilitates the dissociation of ubiquitinated MBP-ODD and VBC from Cul2. MBP-ODD was ubiquitinated with 600 nM UbcH5c and VBC containing HA-pVHL in the presence or absence of the indicated concentration of CSN followed by immunoblotting with an anti-Myc antibody to remove Myc-Cul2. Samples (15%) of Cul2-immunoabsorbed (Cul2 absorbed) material were separated by SDSPAGE followed by immunoblotting with anti-MBP and anti-HA. D, CSN facilitates dissociation of ubiquitinated MBP-ODD from pVHL. MBP-ODD was ubiquitinated as in C followed by immunoblotting with pVHL-immunoabsorbed (pVHL absorbed) material and an equal amount of preabsorption material (Input) were separated by SDS-PAGE followed by immunoblotting with anti-MBP. E, deubiquitylation-defective CSN facilitates the dissociation of ubiquitinated MBP-ODD and pVHL from Cul2. MBP-ODD was ubiquitinated as in C in the presence or absence of CSN containing CSN5-D151N followed by immunoblotting with anti-Myc to remove Myc-Cul2. Samples (15%) of Cul2-immunoabsorbed material and an equal amount of preabsorption material (Input) were separated by SDS-PAGE followed by immunoblotting as in C. F, CSN containing CSN5-D151N also facilitates dissociation of ubiquitinated MBP-ODD from pVHL. MBP-ODD was incubated as in E followed by immunoblotting of pVHL and immunoblotting as in D. Ub, ubiquitin.
CSN-mediated Activation of HIF-α Ubiquitination

MBP-ODD, resulting in accumulation of ubiquitinated MBP-ODD at the gel top when incubated for 60 min (Fig. 1D), and CSN increases the amount of ubiquitinated MBP-ODD in the presence of 600 nM UbcH5c (data not shown). CSN increased the amount of ubiquitinated MBP-ODD in Cul2-depleted mixtures (Fig. 4C, upper gel panel, compare lane 1 with lanes 2 and 3), indicating that CSN sequestered ubiquitinated MBP-ODD from Cul2. CSN also enhanced the dissociation of pVHL from Cul2 (Fig. 4C, lower panel). Immunodepletion of pVHL with a saturating amount of anti-HA antibody (supplemental Fig. 4B) revealed that ubiquitinated MBP-ODD was sequestered from pVHL in the presence of CSN (Fig. 4D). CSN-D151N also induced the dissociation of ubiquitinated MBP-ODD from Cul2 and pVHL (Fig. 4, E and F, and supplemental Fig. 4, C and D). These results indicate that CSN enhances the dissociation of the VBC-Cul2-ubiquitinated MBP-ODD complex into individual components, increasing the efficiency of the ligase complex for recognizing and ubiquitinating MBP-ODD. The de neddylation activity of CSN does not appear to be involved in the dissociation of VBC-Cul2-ubiquitinated substrate complex into VBC, Cul2, and ubiquitinated substrate.

The CSN Complex Facilitates HIF-α Degradation by Inducing the Dissociation of HIF-α from pVHL—We next examined the effect of CSN on the oxygen-dependent degradation of HIF-α mediated by VBC-Cul2 in cells by suppressing the expression of components of CSN with siRNAs specific for these components. Thirty-six hours after the introduction of siRNAs for CSN5 (Fig. 5A) or CSN2 (Fig. 5B), HeLa-VHL cells were cultured in 1% O2 for 6 h to accumulate HIF-α and then in normoxic conditions for the indicated times. In cells transfected with siRNAs for CSN2, expression of both CSN2 and CSN5 were suppressed (Fig. 5C). However, in CSN5 knockdown cells, CSN5 expression was severely suppressed, but expression of CSN2 was not suppressed (Fig. 5C). The precise mechanism underlying this observation is not known, but it might be due to the presence of a CSN subcomplex (21) rather than the stability of the CSN complex. Because siRNA-mediated knockdown of CSN1 strongly suppressed the expression of CSN1, CSN2, and CSN5 (Fig. 5C), CSN5 and CSN2 in HeLa-VHL cells should exist in the CSN complex, and knockdown of these CSN subunits suppresses the expression of intact CSN. Moreover expression of the intact CSN complex is necessary for the function of CSN because transient knockdown of CSN2 by a specific siRNA effectively suppresses the degradation of substrates of Cul1-based CRLs (22). We therefore evaluated the decay of HIF-1α and HIF-2α by harvesting cells at the indicated time points (Fig. 5, A and B). Degradation of both HIF-1α and HIF-2α was delayed in CSN2 and CSN5 knockdown cells compared with cells transfected with control siRNA, suggesting that the intact CSN complex augments the oxygen-dependent degradation of HIF-α. To confirm the effect of CSN observed in our...
in vitro analyses, we examined whether CSN affects pVHL binding to ubiquitinated HIF-1α in HeLa cells (Fig. 5C). HeLa-VHL cells transfected with siRNAs for CSN1, CSN2, or CSN5 were cultured in 1% O₂ for 6 h and then in normoxic conditions for 20 min in the presence of a proteasome inhibitor, MG132, to accumulate prolyl-hydroxylated and ubiquitinated HIF-1α. Knockdown of any of the CSN subunits tested increased the amount of pVHL co-immunoprecipitating with HIF-1α, indicating that the intact CSN complex augments the dissociation of ubiquitinated substrates from VBC-Cul2 in vivo. Taken together, these data indicate that CSN enhances the E3 activity of VBC-Cul2, which increases the efficiency of the ligase complex for recognizing and ubiquitinating prolyl-hydroxylated HIF-1α. CSN then stimulates degradation of the target proteins.

**DISCUSSION**

Neddylation enhances the ubiquitination of HIF-1α by VBC-Cul2 (10). Although CSN removes Ned8 from neddylated cullins, CSN enhances the degradation of substrates of SCF and Cul3-based CRLs in vivo (12–14). Several mechanisms for CSN-mediated activation of the degradation of Cul1- or Cul3-based CRL substrates have been demonstrated recently. First, CSN protects the substrate recognition subunits of CRLs from proteasomal degradation by deubiquitinating the subunits via the CSN-associated deubiquitinating enzyme USP15 (23). CSN has also been shown to stabilize Cul1 and Cul3 from degrada-

**FIGURE 6. Proposed mechanism for CSN-mediated activation of HIF-1α ubiquitination by VBC-Cul2.** VBC recognizing prolyl-hydroxylated HIF-1α binds to Cul2 to form VBC-Cul2-HIF-1α complexes (a) followed by Cul2 neddylation (b). Neddylation of Cul2 enhances the elongation of the polyubiquitin chains conjugated to HIF-1α and recruits CSN, which recognizes polyubiquitinated HIF-1α, to VBC-Cul2 (c). CSN facilitates the dissociation of ubiquitinated HIF-1α and VBC from Cul2 followed by degradation of released HIF-1α by the 26 S proteasome (d). Cul2 deneddylation by CSN5 (e) induces dissociation of CSN from Cul2 (f). Free VBC and Cul2 are recycled to ubiquitinate other HIF-1α molecules (g). Ub, ubiquitin.
cullin deneddylation, interacts with neddylated Cul2 more strongly than with CSN-WT (supplemental Fig. 5) and that CSN-D151N deubiquitinates MBP-ODD more profoundly than CSN-WT (Fig. 3F). Cul2 deneddylation therefore may induce the dissociation of CSN from the ligase complex. We propose that the following mechanism underlies the activation of CSN that leads to substrate degradation (Fig. 6). The binding of VBC to Cul2 induces Cul2 neddylation, which enhances polyubiquitination of the substrates by the VBC-Cul2 ligase complex; CSN is recruited to the neddylated Cul2, recognizes the polyubiquitinated substrates, and then dissociates the ubiquitinated substrates and VBC from Cul2; and finally, Cul2 is deneddylated by CSN5, which dissociates CSN from Cul2.

How does CSN induce dissociation of ubiquitinated substrates from the substrate recognition subunits of CRLs? CSN5 has been reported to bind to HIF-1α (29) and pVHL (30). However, we could not detect binding between CSN and pVHL or MBP-ODD in our assay system (data not shown). Moreover we observed that CSN increased the amount of phospho-14-3-3 interacted with the SCFpVHL ligase (supplemental Fig. 6, compare lanes 5 and 4 with lanes 3 and 2, respectively), indicating that neither the binding of CSN5 to pVHL nor the binding of CSN5 to HIF-1α is involved in the CSN-mediated increase of the amount of ubiquitinated MBD-ODD. Instead we hypothesize that CSN recognizes the polyubiquitin chain directly and removes polyubiquitinated MBP-ODD from pVHL because CSN can bind to tetraubiquitin (19). Because we failed to observe CSN binding to ubiquitinated MBP-ODD by co-immunoprecipitation in our in vitro assays (data not shown), the binding of CSN to polyubiquitin may be weak. We also found that a substoichiometric amount of CSN (30 nM) is sufficient for dissociation of ubiquitinated MBP-ODD from pVHL (200 nM; Fig. 4, C and D), indicating that CSN binds to polyubiquitin only temporally, but the temporary binding may be sufficient for inducing dissociation of ubiquitinated substrates from CRLs.

In our assay system, neither Cul2 neddylation nor the deneddylation activity of CSN appeared to be critical for enhancing the efficiency of CRLs in recognizing and ubiquitinating substrates; this might contradict our hypothesis (Fig. 6). We suspect that both polyubiquitin chains conjugated to substrates and Cul2 neddylation may be necessary to cooperatively enhance the binding of CSN to CRLs. If this is the case, CSN could bind to CRLs even without cullin neddylation or the deneddylation activity of CSN, and the binding of CSN to CRL may depend on the conjugation of polyubiquitin chains to substrates by CRL in our in vitro assays. Our observation that the length of the conjugated polyubiquitin chain is critical for the enhancement of the number of ubiquitinated MBP-ODD molecules by a single VBC-Cul2 ligase may support this idea.

It has been shown that CAND1 is involved in the neddylation and complex formation cycles of Cul1- and Cul3-based CRLs (31, 32). However, we doubt that CAND1 is involved in CSN-induced enhancement of MBP-ODD ubiquitination because CAND1 either cannot bind, or can only bind very weakly, to Cul2 (31). Our preliminary analysis suggested that the addition of CAND1 to our in vitro assays has no effect on the CSN-mediated increase in the amount of ubiquitinated MBP-ODD (data not shown). Because we used a CSN complex purified from U2OS cells, we suspect that the effect of CSN may be mediated by currently unidentified CSN-associated molecules. Further characterization of the molecules that were co-purified with the CSN complex will clarify the components involved in the dissociation of polyubiquitinated substrates.

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