Hypoxia-inducible Factor α Subunit Stabilization by NEDD8 Conjugation Is Reactive Oxygen Species-dependent*

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Ji-Hye Ryu†1, Shan-Hua Li‡§1, Hyoung-Sook Park‡, Jong-Wan Park§, ByungLan Lee¶, and Yang-Sook Chun¶§2
From the Departments of †Physiology, ‡Pharmacology, and §Anatomy, Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea and the ¶Department of Basic Medical Sciences, Medical College, Xiamen University, Xiamen, 361005 Fujian, China

Hypoxia-inducible factor α proteins (HIF-αs) are regulated oxygen dependently and transactivate numerous genes essential for cellular adaptation to hypoxia. NEDD8, a member of the ubiquitin-like family, covalently binds to its substrate proteins, and thus, regulates their stabilities and functions. In the present study, we examined the possibility that the HIF signaling is regulated by the neddylation. HIF-1α expression and activity were inhibited by knocking down APPBP1 E1 enzyme for NEDD8 conjugation but enhanced by ectopically expressing NEDD8. HIF-1α and HIF-2α were identified to be covalently modified by NEDD8. NEDD8 stabilized HIF-1α even in normoxia and further increased its level in hypoxia, which also occurred in von Hippel-Lindau (VHL) protein- or p53-null cell lines. The HIF-1α-stabilizing effect of NEDD8 was diminished by antioxidants and mitochondrial respiratory chain blockers. This suggests that the NEDD8 effect is concerned with reactive oxygen species driven from mitochondria rather than with the prolyl hydroxylase (PHD)/VHL-dependent oxygen-sensing system. Based on these findings, we propose that NEDD8 is an ancillary player to regulate the stability of HIF-1α. Furthermore, given the positive role played by HIF-α in cancer promotion, the NEDD8 conjugation process could be a potential target for cancer therapy.

Hypoxia-inducible factors (HIFs)3 play crucial roles in tumor adaptation to hypoxia and angiogenesis by up-regulating numerous genes (1). HIF family members are composed of α (HIF-1α and HIF-2α) and β (also known as aryl hydrocarbon receptor nuclear translocator (ARNT)) subunits. The HIF-αs are tightly regulated by oxygen tension and function as prime transactivating factors, whereas aryl hydrocarbon receptor nuclear translocator is constitutively expressed and assists HIF-α binding to DNA. Under normoxic conditions, two proline residues (Pro-402 and Pro-564) in the oxygen-dependent degradation domain (ODDD) of HIF-1α are hydroxylated by prolyl hydroxylases (PHD1–3) (2); subsequently, HIF-1α is ubiquitinated by von Hippel-Lindau protein (pVHL) and finally degraded by 26 S proteasome (3). However, this hydroxylation is limited under hypoxic conditions, which stabilizes HIF-αs. Given the essential roles played by HIFs in tumor promotion, the HIF inhibition has become a frontline topic in research on new cancer therapies (4).

NEDD8 (neural precursor cell-expressed developmentally down-regulated 8) is conserved in eukaryotes and is ubiquitously expressed in most mammalian tissues. As NEDD8 is structurally similar to ubiquitin, it is classified as a member of the ubiquitin-like family (5). Furthermore, like ubiquitin, NEDD8 conjugates to its substrate proteins, which is named “neddylation,” via a sequential process involving activation, conjugation, and ligation. Neddlylation requires a unique set of conjugating enzymes, namely NEDD8-activating E1 complex, which is composed of APPBP1 and UBA3, NEDD8-conjugating E2 enzyme (UBC12), and various NEDD8-ligase E3 enzymes (6). Functionally, neddlylation is essential for cell viability, development, and responses to stress, and thus, disorganized neddlylation is regarded to be associated with the pathogeneses of neurodegenerative and neoplastic diseases (7, 8). Accordingly, neddlylation is viewed as a potential therapeutic target. Recently, an E1 inhibitor MLN4924 was found to induce cell cycle arrest and apoptosis in cultured cancer cells and to effectively inhibit tumor growth in mice (9), which aroused much interest in the neddlylation-targeting cancer therapy.

The ubiquitin-proteasome system is viewed as a promising target for the development of anticancer agents, and several proteasome inhibitors have been subjected to clinical trials in patients with multiple myeloma and in patients with some solid tumors (10). Proteasome inhibitors induce tumor cell death and inhibit tumor adaptation to hypoxia by inactivating HIF-1 (11), and thus, the double targeting of tumor cells and their microenvironments with proteasome inhibitors might synergistically inhibit tumor growth in vivo. Likewise, neddlylation is now being introduced as a novel target for anticancer treatment. However, the role of neddlylation in tumor growth has only been investigated in the context of tumor cell death and not with respect to tumor adaptation to hypoxia. Therefore, in the present study, we tested the possibility that NEDD8 is an ancillary player to regulate the stability of HIF-1α. It is hoped that this work provides a rationale for the development of neddlylation-targeting cancer therapy.
**HIF-α Stabilization by Neddylation**

**EXPERIMENTAL PROCEDURES**

**Antibodies and Chemicals**—Antibodies against APPBP1 (Abcam, Cambridge, UK), NEDD8 (Cell Signaling Technology, Danvers, MA), FLAG tag (Sigma-Aldrich), HA tag (Roche Applied Science), and HIF-2α (Novus Biologicals, Inc, Littleton, CO) were obtained from the indicated companies. Anti-β-tubulin and anti-lamin B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Mn-SOD antibody was purchased StressGen (Victoria, British Columbia, Canada). Anti-HIF-1α antibody was raised against ODDD of human HIF-1α in rabbits (12). MG132, PD98059, U0216, and LY294002 were purchased from Alexis Biochemicals (Lausen, Switzerland), and cycloheximide, CBZ-LLN, N-acetyl cysteine, Tiron, Trolox, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), myxothiazol, antimycin A, Geldanamycin, 2′,7′-dichlorofluorescein diacetate (DCFDA), Z-VAD-FMK, EGTA, and other chemicals were purchased from Sigma-Aldrich.

**Cell Culture**—HEK293 (human embryonic kidney) cell line was obtained from the American Type Culture Collection. VHL(+/+) RCC4 (kidney cancer), and p53(+/-; -/-) HCT116 (colon cancer) cell lines were obtained from the European Collection of Animal Cell Cultures. These cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS. Cells were incubated in either 5% CO2, 20% (normoxic) or 1% (hypoxic) O2 atmospheres at 37 °C.

**Plasmids, siRNA, and Transient Transfection**—Plasmids of hif1α and hif2α were constructed by PCR amplification and recombination into HA-tagged pcDNA3 vector. Oxygen independently stable HIF-1α mutant (pHA-P402A, P564A) was made using a PCR-based mutagenesis kit (Stratagene, Cedar Creek, TX). NEDD8 expression plasmid was constructed by reverse transcription-PCR (RT-PCR) and blunt-end ligation in-frame with FLAG- or His6-tagged pcDNA3 vector. Mutant NEDD8 (NEDD8-ΔGG), which is unable to conjugate with target proteins, was made by carboxyl-terminal deletion from Gly-75. An mnsod expression vector, pcDNA3, containing a human mnsod cDNA insert (780 bp) and an appbp1 targeting siRNAs were synthesized by Samchully Pharm (Gyeonggi-do, Korea). The target region of appbp1 (GenBank™ number NM_003905) correspond to nucleotides from 996 to 1014 (si-hif1α) and from 667 to 685 (si-hif2α) of the coding region. The sequences of rank1 (receptor for activated K=ase-1), sart1 (squamous cell carcinoma antigen recognized by T cells), and klf2 (Kruppel-like factor 2) siRNAs correspond to nucleotides from 133 to 159 (si-RACK1) of the coding region of the rank1 gene (NM_006098), from 1845 to 1869 (si-HAF) of the sart1 gene (NM_005146), from 839 to 863 (siKLF2) of the klf2 gene (NM_016270), and from 581 to 606 (si-VHL) of the vhl gene (NM_000551), respectively. The sequence of control siRNA (si-Con) was 5′-CAAGACCCGCGCCAGGUGAUU-3′. Plasmids and siRNAs were transiently transfected into cells using the calcium phosphate or Lipofectamine (Invitrogen) method. 48 h after transfection, cells were harvested and used in the experiments.

**Semiquantitative RT-PCR**—Total RNAs were isolated from cultured cells using TRIzol (Invitrogen). To quantify vascular endothelial growth factor (VEGF), PDK1, and β-actin mRNA levels, we used the semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) method and autoradiography, as described previously (13).

**Luciferase Assay**—Luciferase reporter genes, containing hypoxia responsive element of the human vega gene and the erythropoietin (epo) enhancer region, were constructed as described previously. HEK293 cells were cotransfected with 1 μg each of reporter gene, 50 nm siRNAs, and β-gal plasmid using the calcium phosphate method. After stabilization for 48 h, cells were incubated under either normoxic or hypoxic conditions for 16 h. Luciferase activities were measured using a Biocounter M1500 luminometer (Lumac) and normalized to β-gal activities.

**Immunoblotting and Immunoprecipitation**—Total cell lysates in a SDS sample buffer were separated on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked from nonspecific proteins with 5% skim milk in TTBs (Tris-buffered saline containing 0.1% Tween 20) for 30 min and incubated overnight with a primary antibody diluted in 1:1000 to 5000 in the blocking solution. Then, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:5000 in the blocking solution) for 1 h, and blots were visualized using an ECL Plus kit (Amersham Biosciences). For immunoprecipitation, cell lysates (1 mg of protein) were incubated with 5 μl of antibody for 2 h and then incubated with 10 μl of protein A/G-Sepharose beads (GE Healthcare) for 4 h at 4 °C. After washing, the immunoprecipitated proteins were eluted in the SDS sample buffer and subjected to SDS-PAGE and Western blotting.

**Identification of His6-tagged NEDD8 Conjugates**—Identification of NEDD8 conjugation was performed and modified based on the description in Jaffray and Hay (14). After transfection of plasmid expressing His6-tagged NEDD8 or NEDD8ΔGG, cells were divided into two dishes. One was lysed with SDS sample buffer and analyzed by Western blotting to confirm the expression level of proteins (input samples). The other was lysed by adding denaturing buffer (6 M guanidine hydrochloride, 0.1 M NaH2PO4/Na2HPO4, 0.01 M Tris–Cl (pH 8.0), 10 mM imidazole and 10 mM β-mercaptoethanol) directly to the cells. The lysates were mixed with Ni2+-NTA-agarose beads (Qiagen, Valencia, CA), prewashed with lysis buffer, and rotated for 4 h at room temperature. The beads were successively washed for 5 min in each step with the following solutions: lysis buffer (pH 8.0); washing buffer (pH 8.0) (8 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris/HCl, pH 8.0, plus 20 mM imidazole, 10 mM β-mercaptoethanol); washing buffer (pH 6.3) plus 0.2% Triton X-100; and washing buffer (pH 6.3) plus 0.1% Triton X-100. Then, the beads were eluted with SDS sample buffer and analyzed by Western blotting.

**Preparation of Nuclear Extract**—Cells were harvested, washed twice with ice-cold PBS, resuspended in buffer A (20 mM Tris, pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, protease inhibitor mixture, and 1 mM Na3VO4), and cooled on ice. After adding 0.6% Nonidet P-40, lysed cells were centrifuged, and the supernatants were collected as the cytosolic fraction. Nuclear pellets were resuspended in a buffer B (400 mM NaCl and 5% glycerol in buffer A)
and incubated at 4 °C for 30 min. After centrifugation, the supernatants were kept as the nuclear fraction.

Assay of ROS—293T cells in the experimental conditions were washed by prewarmed PBS, culture media were replaced with a Hank’s balanced salt solution that had been preincubated at 37 °C in 5% CO₂ atmosphere, and then cells were treated with DCFDA (50 μM) for 30 min in the dark and detached with trypsin-EDTA solution. After a brief washing, the oxidized form of DCFDA, fluorescent dichlorofluorescein, was excited at 488 nm and detected at 530 nm wavelength, using a FACStar flow cytometer.

Statistical Analysis—All data were analyzed using Microsoft Excel 2002 software, and results are expressed as means ± S.D. We used the unpaired Student’s t test to compare reporter activities. Differences were considered statistically significant at the p < 0.05 level. All statistical tests were two-sided.

RESULTS

Neddylation-dependent Activation of Hypoxic Signaling due to HIF-1α Stabilization—To examine whether the HIF-dependent hypoxic signaling is regulated by neddylation, we inhibited the initial step of neddylation by knocking down APPBP1. Under hypoxic conditions, VEGF and PDK1 mRNA levels increased in Hep3B cells, which was reversed by appbp1 siRNAs (Fig. 1A, upper). We also found that HIF-1α is down-regulated by appbp1 siRNAs (Fig. 1A, lower). Furthermore, vegfa promoter and epo enhancer reporter analyses showed that the APPBP1 inhibition attenuated the transcriptional activity of HIF-1 under hypoxic conditions (Fig. 1B). To understand the mechanism underlying HIF-1α suppression by APPBP1 knockdown, we checked the synthesis and degradation of HIF-1α protein. Newly synthesized HIF-1α accumulated after blocking its degradation with MG132, but the rate of HIF-1α synthesis was constant regardless of APPBP1 expression (Fig. 1C, upper). The rate of HIF-1α degradation was analyzed by reoxygenating cells having been subjected to hypoxia. Fig. 1D shows that the HIF-1α degradation was accelerated about three times in APPBP1 knockdown cells. These results suggest that neddylation is required for HIF-1α stabilization in hypoxia.

NEDD8 Conjugation of HIF-αs—To investigate whether NEDD8 directly regulates HIF-α, we checked HIF-1α expression and activity in HEK293 cells that had been transfected with FLAG-NEDD8 or FLAG-NEDD8ΔGG (conjugation-defective due to Gly-75/76 deletion) plasmid. Endogenous HIF-1α level and activity were increased by NEDD8 overexpression, but not by NEDD8ΔGG (Fig. 2A). In addition, ectopically expressed HIF-1α was also stabilized by NEDD8 (Fig. 2B). We examined the interaction between HIF-1α and NEDD8 and found that the two proteins are co-precipitated by each other in hypoxic HEK293 cells (Fig. 2C). Then is HIF-1α conjugated with NEDD8? HEK293 cells, which had been transfected with His-NEDD8 or His-NEDD8ΔGG, were subjected to hypoxia, and proteins were pulled down by Ni²⁺ under a denaturing condition. Endogenous HIF-1α was induced in conjugation with NEDD8, but not by NEDD8ΔGG (Fig. 2D). Also, ectopically expressed HIF-αs were identified to be conjugated with NEDD8 (Fig. 2E).

Calculation of Neddylated Form in the Stabilized HIF-1α by Hypoxia—To determine the portion of neddylated HIF-1α under hypoxia, we measured how much HIF-1α is reduced by silencing APPBP1. HIF-1α levels were determined by immunoblotting (upper panel) and quantified using the ImageJ program (lower panel). HIF-1α stability depends on neddylation. HEK293 cells were transfected with siRNAs and stabilized for 2 days and then treated with 100 μM cycloheximide for 1 h to remove the remaining HIF-1α completely. After washing out cycloheximide, cells were further incubated for the indicated times with MG132. Protein levels were analyzed by Western blotting (upper panel) and quantified using the ImageJ program (lower panel). D, HIF-1α stability depends on neddylation. HEK293 cells were transfected with siRNAs and stabilized for 48 h. Cells were exposed to 4 h of hypoxia and then subjected to normoxia for indicated times. Protein levels were analyzed by Western blotting (upper panel) and quantified using the ImageJ program (lower panel). Half-lives (t₁/₂) of HIF-1α were calculated from the slopes of the first-order decay curves. Points represent the means ± S.D. of three experiments.
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**FIGURE 2. Neddylation stabilizes and activates HIF-1α.** A, NEDD8 increases the expression and activity of endogenous HIF-1α. HEK293 cells were transfected with the indicated plasmids. Protein expressions were analyzed in the nuclear fractions by Western blotting. Lamin B was analyzed as a loading control for nuclear proteins (upper panel). Luciferase activities (means ± S.D., n = 8) are presented as relative values versus the pcDNA control. *, p < 0.01 versus the pcDNA control (lower panel). B, ectopic HIF-1α proteins are stabilized by NEDD8. HA-HIF-1α or HA-HIF-2α (1 μg) were cotransfected with FLAG-NEDD8 or FLAG-NEDD8ΔGG (3 μg) into HEK293 cells. After stabilization for 48 h, cell lysates were analyzed by Western blotting. C, endogenous HIF-1α associates with NEDD8 under hypoxia. HEK293 cells were incubated in normoxia (N) or hypoxia (H) for 8 h. Cell lysates were immunoprecipitated using anti-HIF-1α or anti-NEDD8, and coprecipitated proteins were analyzed by Western blotting (IB) using anti-NEDD8 or anti-HIF-1α, respectively. D, neddylation of endogenous HIF-1α. His-NEDD8ΔGG plasmid was transfected into HEK293 cells, and cells were lysed. His-NEDD8-conjugated proteins were bound to Ni²⁺-NTA-agarose beads under denaturing conditions and eluted with a SDS sample buffer. Proteins were analyzed by Western blotting. E, neddylations of ectopically expressed HIF-1α and HIF-2α. After cotransfection with HIF-1α/2α and His-NEDD8ΔGG plasmids, HEK293 cells were lysed under denaturing conditions. His-NEDD8-conjugated HIF-1α/2α were isolated with Ni²⁺-NTA-resin. Proteins were analyzed by Western blotting. F, calculating neddylated HIF-1α using qppb1 siRNA. HEK293 cells were transfected with siRNAs and stabilized for 48 h, and then cells were exposed to 4 h of hypoxia. Protein levels were analyzed by Western blotting (upper panel) and quantified using ImageJ (lower panel). The amount of HIF-1α stabilized by neddylation was calculated as the difference between si-Con and si-APPB1 groups. Bars represent the means ± S.D. of six experiments. G, calculating neddylated HIF-1α using qppb1 siRNA. HEK293 cells were transfected with siRNAs and stabilized for 48 h, and then cells were exposed to 4 h of hypoxia. Cell lysate was separated into six tubes, and Ni²⁺-NTA-agarose was added into the tubes at the indicated amounts. The mixtures were incubated for 2 h, and then Ni²⁺-bound proteins were purified, and input proteins were analyzed by Western blotting (WB). FL, full length. B, neddylation of HIF-1α PAS-B domain. Full-length (1–826), ΔN1 (201–826), or ΔN2 (401–826) of HA-tagged HIF-1α plasmid was cotransfected with His-NEDD8, and His-NEDD8-conjugated proteins were purified and analyzed by Western blotting.
the neddylation site of HIF-1α is present between amino acids 201 and 400, which corresponds to the PAS-B domain.

**HIF-α Stabilization by Neddylation Occurs Independently of PHD, VHL, and p53**—To understand how HIF-α is stabilized by neddylation, we first checked the involvement of prolyl hydroxylation in the process. However, a stable HIF-1α mutant (sHIF-1α), which lacks both hydroxylated prolines Pro-402 and Pro-564, was still stabilized and covalently bound by NEDD8 (Fig. 4, A and B). Next, we examined whether neddylation blocks the pVHL-mediated degradation of HIF-1α, but the expressions of HIF-1α and its downstream genes were suppressed by APPBP1 knockdown even in VHL-defective RCC4.

**FIGURE 4.** HIF-1α stabilization by neddylation is independent of PHD, pVHL, and p53. A, NEDD8 stabilizes HIF-1α regardless of proline hydroxylation. HEK293 cells were cotransfected with HA-tagged stable HIF-1α (HA-sHIF-1α; P402A/P564A) plasmid and FLAG-NEDD8/ΔGG plasmid. After 24 h of stabilization, protein levels were analyzed by Western blotting. B, neddylation of stable HIF-1α mutant. HA-sHIF-1α plasmid was cotransfected with His-NEDD plasmid, and then HEK293 cells were lysed under denaturing conditions. His-NEDD8-conjugated HIF-1α was isolated with Ni²⁺ affinity resin, and proteins were analyzed by Western blotting.

C, HIF-1α neddylation and stabilization are VHL-independent. VHL+/− and RCC4+/− cells were transfected with 50 nM of control or appbp1 siRNA. After normoxic or hypoxic incubation for 16 h, the mRNAs of VEGF, PDK1, and β-actin were analyzed by semiquantitative RT-PCR and autoradiography. Total proteins in cell lysates were analyzed by immunoblotting. D, neddylation of ectopic HIF-1α in VHL+/− and RCC4+/− cells. After cotransfection with HIF-1α and His-NEDD8 plasmids, cells were lysed under denaturing conditions. His-NEDD8-conjugated HIF-1αs and input proteins were analyzed by Western blotting.

E, HIF-1 activation by neddylation occurs even in VHL knockdown cells. After transfection with si-VHL, HEK293 cells were cotransfected with EPO reporter, si-APPBP1, and β-gal plasmids. After normoxic or hypoxic incubation for 16 h, luciferase activities were measured and normalized to β-gal activity. Results (means ± S.D., n = 8) are presented as relative values versus the normoxic si-Con groups. †, p < 0.01 versus the normoxic si-Con; *, p < 0.01 versus the hypoxic si-Con.

F, HIF-1α neddylation and stabilization are not affected by p53. p53+/+ and p53−/− HCT116 cells were transfected with HIF-1α and His12-NEDD8. HIF-1α expression and neddylation were analyzed by Ni²⁺-binding and Western blotting.
FIGURE 5. HIF-1\(\alpha\) neddylation depends on mitochondrial ROS. A, the HIF-1\(\alpha\) stabilization by neddylation is abolished by antioxidants and mitochondrial inhibitors. After cotransfection with HA-HIF-1\(\alpha\) and His-NEDD8, HEK293 cells were incubated in the normoxia or hypoxia with antioxidants (left panel) or mitochondrial respiratory inhibitors (right panel). Proteins indicated were immunoblotted (upper panel), and neddylated HIF-1\(\alpha\) was isolated with Ni\(^{2+}\) affinity resin under denaturing conditions (lower panel). Agents used are: N, 5 mM N-acetyl cysteine; T, 1 mM Tiron; Tr, 10 \(\mu\)M Trolox; D, 600 \(\mu\)M DIDS; M, 100 \(\mu\)M myxothiazol; A, 10 \(\mu\)M antimycin A; B, intracellular ROS levels. After transfection with His-NEDD8, HEK293 cells were incubated in the normoxia or hypoxia with the indicated agents. Proteins were analyzed by Western blotting (lower panel), and ROS levels were detected by flow cytometry. Data (\(n = 4\)) are plotted as relative values to the normoxic control (upper panel). Agents used are: N, 5 mM N-acetyl cysteine; D, 600 \(\mu\)M DIDS; PQ, 100 \(\mu\)M Paraquat. C, the HIF-1\(\alpha\) stabilization by neddylation is abolished by Mn-SOD. After transfection with His-NEDD8 or and Mn-SOD, HEK293 cells were incubated under indicated conditions, and then protein levels (lower panel) and ROS levels (upper panel) were analyzed by the aforementioned methods. ROS data (\(n = 7\)) are plotted as relative values to the normoxic control, \(p < 0.05\) versus the normoxic control.

**DISCUSSION**

Neddylation is now being introduced as a novel target for anticancer treatment. However, the role of neddylation in tumor growth has only been investigated in the context of tumor cell death and not with respect to tumor adaptation to hypoxia. In the present study, we found that the hypoxic gene regulation is ensured by neddylation of HIF-\(\alpha\) and that the neddylation is required for the stabilization of HIF-\(1\alpha\) by mitochondrial ROS.

The oxygen-dependent degradation of HIF-1\(\alpha\) is initiated by the hydroxylation of prolines 402 and 564. Then, pVHL targets the hydroxylated HIF-1\(\alpha\) and recruits elongin B/C, Cul2, and Rbx1, which forms the VBC-Cul2 E3-ubiquitin ligase complex to ubiquitinate HIF-1\(\alpha\) (19). Of these components, pVHL and Cul2 have been identified to be covalently conjugated by NEDD8. In functional aspects, neddylated pVHL cannot recruit Cul2 to HIF-1\(\alpha\) and rather preferentially interacts with fibronectin (20). Accordingly, the pVHL neddylation could negatively regulate the ubiquitination of HIF-1\(\alpha\), but this possibility has not been investigated. On the contrary, Cul2 is neddylated after recruited by pVHL, and the neddylated Cul2 facilitates the polyubiquitination of HIF-1\(\alpha\) (21–23). Therefore, the net effect of neddylation on HIF-1\(\alpha\) stability is obscure. Nonetheless, Ohh et al. (24) suggested that the neddylation process is required for the ubiquitination and subsequent degradation of HIF-1\(\alpha\). They demonstrated that HIF-1\(\alpha\) is up-regulated at a non-permissive temperature in CHO cells having a temperature-sensitive mutation of APPBP1, which contradicts our results. Therefore, we checked whether or not the effects of NEDD8 on HIF-1\(\alpha\) are linked with pVHL-mediated HIF-1\(\alpha\) ubiquitination. Consequently, HIF-1\(\alpha\) expression and activity were still regulated by NEDD8 in VHL-defective cells. Given our results and a report of Ohh et al. (24), it is concluded that the direct neddylation of HIF-1\(\alpha\) may overcome the HIF-1\(\alpha\)-destructive effect of neddylated Cul2. The reciprocal regulations of HIF-1\(\alpha\) by neddylation remain to be investigated.

HIF-1\(\alpha\) and p53 reciprocally regulate each other, that is, HIF-1\(\alpha\) stabilizes p53 but p53 destabilizes HIF-1\(\alpha\) (25, 26). In addition, p53 is known to be inactivated by neddylation via Mdm2 (27). Therefore, HIF-1\(\alpha\) could be indirectly stabilized by p53 neddylation. To test this possibility, we examined HIF-1\(\alpha\) neddylation in p53\(\text{(+/-)}\) HCT116 cell lines, and found that HIF-1\(\alpha\) in both cell lines was modified and stabilized by NEDD8. Therefore, the HIF-1\(\alpha\) neddylation and stabilization are unlikely to be p53-dependent.
Heat-shock protein 90 (Hsp90) interacts with the PAS-B domain and stabilizes HIF-1α, which was identified to occur independently of pVHL (28). Therefore, we examined whether Hsp90 is involved in the NEDD8-dependent stabilization of HIF-1α. HEK293 cells, which had been transfected with FLAG-tagged NEDD8 or ΔGG plasmin, were treated with geldanamycin (an Hsp90 inhibitor) in normoxia or in hypoxia. However, geldanamycin failed to attenuate the NEDD8-dependent stabilization of HIF-1α (data not shown), which suggests that Hsp90 does not participate in the NEDD8 action. Besides Hsp90, several other molecules have been reported to regulate the stability of independently of HIF-1α VHL by directly targeting the N terminus of HIF-1α. For example, receptor for activated C kinase-1 (RACK1) serves as a scaffold protein that recruits elongin C to HIF-1α independently of oxygen, which leads to the ubiquitination of HIF-1α (29). Furthermore, the HAF E3-ubiquitin ligase and Kruppel-like factor 2 (KLF2) have been reported to promote the degradation of HIF-1α independently of VHL (30, 31). Therefore, we examined the involvement of RACK1, HAF, or KLF2 in the NEDD8 stabilization of HIF-1α using their siRNAs, but none of the siRNAs reversed the effect of NEDD8 on HIF-1α stability (data not shown). We also checked the possibility that NEDD8 conjugation inhibits the HIF-1α degradation by other proteolytic enzymes. Even when cells were treated with proteasome inhibitors (MG132, CBZ-LLN), a caspase inhibitor (Z-VAD-FMK), a metalloprotease inhibitor (EGTA), or a lysosome inhibitor (NH₄Cl), HIF-1/2α proteins were induced by NEDD8 (data not shown). In addition, various kinase inhibitors, such as PD98059 (ERK inhibitor), U0126 (MEK inhibitor), and LY294002 (PI3K inhibitor), showed no effect on the NEDD8-dependent HIF-1α stability (data not shown). Because these trials failed, we investigated the possible role of ROS in the stabilization of neddylated HIF-1α.

The role of ROS in hypoxic signaling had been controversial before Chandel et al. (15, 32) clearly demonstrated the role of mitochondrial ROS in HIF-1 signaling in hypoxia. Later, three reports (16–18) further supported that mitochondrial ROS are required for the normoxic and hypoxic stabilization of HIF-1α. Nevertheless, the precise mechanism by which ROS stabilize HIF-1α is still unclear. Therefore, we tested whether the NEDD8 stabilization of HIF-1α is linked with mitochondrial ROS. The NEDD8 effect on HIF-1α stabilization was abolished by antioxidants, respiratory chain inhibitors, DIDS, and Mn-SOD. All of the results support that the HIF-1α neddylation is required for mitochondrial ROS to stabilize HIF-1α. Then how do NEDD8 and ROS cooperatively stabilize HIF-1α? In response to this question, we can hypothesize two possible scenarios; that is, “ROS mediate the neddylation of HIF-1α” and “Neddylated HIF-1α is favorably stabilized by ROS.” The precise mechanism remains open in the present study.

In conclusion, NEDD8 conjugation is likely to augment the HIF-driven hypoxic signaling. Moreover, this action of NEDD8 may have a greater impact on cell survival under circumstances where hypoxia and oxidative stress coexist. Tumors commonly face hypoxia and oxidative stress, and they should overcome these microenvironmental factors to keep growing. Therefore, the neddylation inhibition could be a potential strategy to control tumor growth by targeting the microenvironment. Also, it would be worthwhile evaluating the anti-HIF and anti-angiogenic actions of neddylation inhibitors before they are subjected to clinical studies.

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