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Cobalt- and Nickel-Binding Property of Cullin-2

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Treatment with divalent metal ions such as cobalt (Co^{2+}) or nickel (Ni^{2+}) result in the stabilization of hypoxia-inducible factor-1α (HIF1α). Recently, HIF1α was shown to be ubiquitinated by an E3-ligase complex and be subsequently targeted for proteasomal degradation. In this study, we demonstrated that Co^{2+} and Ni^{2+} specifically bind to cullin-2. Mutant analysis revealed that cullin-2 possesses at least three sites for the binding. Furthermore, fluorescence spectroscopy revealed that only Co^{2+} and Ni^{2+} have the binding activity to cullin-2, but other metal ions, including Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, did not. Finally, we found that Co^{2+} and Ni^{2+} do not bind to any components of the E3-ligase other than cullin-2, suggesting that cullin-2 is a key target of Co^{2+} and Ni^{2+}. Interestingly, Co^{2+} did not affect the complex formation of the ligase, suggesting that the metal binding to cullin-2 affects the function, but not the assembly of the E3-ligase.

Hypoxia induces numerous genes involved in angiogenesis, glucose transport, glycolysis, erythropoiesis, and cathecholamine metabolism. The induction is mediated by a transcription factor known as hypoxia-inducible factor (HIF), which is composed of HIFα and HIFβ subunits (1). HIFα subunits are expressed at low levels owing to their rapid degradation in normoxia but are stabilized in hypoxic condition (2). Recently, HIFα was shown to be ubiquitinated by an E3 ubiquitin ligase, called SCF-like complex, and degraded by the proteasome pathway (3, 4). The SCF-like complex is composed of von Hippel-Lindau gene product (pVHL), elongin B, elongin C, cullin-2 (Cul2), ROC1, and NEDD8. Most recently, workers reported that HIFα is modified by prolyl hydroxylation and targeted to pVHL for ubiquitination (5–7). Because the hydroxylation requires oxygen, this protein modification is thought to play a key role in stabilization of HIFα by hypoxic condition.

Like hypoxic stress, divalent metal ions, such as Co^{2+} and Ni^{2+}, can stabilize HIFα, resulting in HIF-dependent transcription (1, 8, 9). The mechanism of the stabilization mediated by Co^{2+} and Ni^{2+} is still unclear. In this report, we demonstrated the direct binding of these metal ions to cullin-2 and proposed a model to explain HIFα stabilization mediated by Co^{2+} or Ni^{2+} treatment.

MATERIALS AND METHODS

Cell lines and culture conditions. Human embryonic kidney (HEK) 293 cells and COS-M6 cells were used as described previously (10). Human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (Portland, OR). Using a tissue culture dish coated with gelatin, HUVEC were maintained in M199 medium supplemented with 15% fetal calf serum, 0.1 mg/ml heparin (Sigma, St. Louis, MO), 0.05 mg/ml endothelial cell mitogen (Biomedical Technologies, Stoughton, MA) and antibiotics.

Antibodies. Mouse anti-RH monoclonal antibody (mAb) specific for the amino sequence RGSHHHH was purchased from QIAGEN (Santa Clara, CA). Mouse mAb 16B12 (Covance, Richmond, CA) is an antibody to the peptide sequence YPYDVPDYA of influenza hemagglutinin (HA). Rabbit polyclonal anti-actin antibody (specific for the C-terminal actin fragment) was purchased from Sigma. GST-12, a mouse mAb specific for glutathione-*S*-transferase (GST), was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human HIF1α mAb specific for amino acids 610–727 was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-Cul2 antibody (Catalog No. 51-2000, recognizing the C-terminus of human cullin-2) was purchased from Zymed Laboratories (South San Francisco, CA).

Plasmid construction and transfection. To express proteins tagged with epitope at the N-terminus in COS-M6 cells, pcDNA3/3HA-N or pcDNA3/3RH-N was used as previously described (11, 12). The human cDNAs of Cul1 (12), Cul2 (13), Cul3 (GenBank Accession No. NM003590), Cul4A (Accession No. AF077188), Cul5 (Accession No. AF327710), Uba3 (12), pVHL (11), elongin B (11), elongin C (11), NEDD8 (14), and ROC1 (Accession No. AF140598) were amplified by polymerase chain reaction (PCR) using appropriate primers from the cDNA library of human testis or brain (Clontech, Palo Alto, CA). These cDNAs were inserted into the aforementioned plasmid vectors, and transfected into COS-M6 cells using FuGENE 6 (Roche Molecular Biochemicals). The transfected cells were harvested for

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Western blotting or precipitation with metal-ion-coated beads 20 h after transfection.

Precipitation with metal-ion-immobilized beads. To precipitate Co^{2+}- or Ni^{2+}-binding proteins and proteins tagged with RH-epitope (RGSHHHHHH), we used Co^{2+}-immobilized resin beads (TALON beads, CLONTECH) or nickel-charged agarose resin beads (Invitrogen) as described previously (12–14).

Expression and purification of GST-fusion proteins. The cDNAs of Cul2 wild type, Cul2 mutants, pVHL, elongin B, elongin C, NEDD8, and ROC1 were subcloned into a bacterial expression vector, pGEX-2TK or pGEX-5X-3 (Amersham Pharmacia Biotech, Piscataway, NJ). The plasmid vectors were transformed into *Escherichia coli* BL 21 (Stratagene, La Jolla, CA) and GST-fusion proteins were purified from the bacteria as described previously (15).

For the precipitation of GST-fusion proteins by Co^{2+}- or Ni^{2+}-immobilized resin beads, the bacterial pellet was resuspended in 150 μl of the lysis buffer for beads (12). The bacteria were lysed on ice by mild sonication and centrifuged at 14,000 rpm for 10 min at 4°C by Eppendorf centrifuge. The bacterial supernatants were mixed with 150 μl of the lysis buffer and incubated for 1 h at room temperature with 20 μl of beads that had been previously washed three times and resuspended (final concentration, 1:1 (v/v)) in the lysis buffer. The beads were then washed once with the lysis buffer, followed by washing twice with the washing buffer for beads described previously (12). Finally, the beads were washed twice with PBS and treated at 45°C for 1 h in a sample buffer containing 2% SDS and 5% β-mercaptoethanol. The solubilized proteins were analyzed by Western blotting using the mouse anti-GST mAb, GST-12.

Western blotting. Western blotting was performed as described previously (12).

Generation of adenovirus vector. To express FLAG-tagged pVHL in HUVEC, Adeno-X expression system was purchased from Clontech and generated the adenovirus. The HEK 293 cells were cultured, and low-titer recombinant adenovirus was prepared from the growth medium. High-titer viral stocks were prepared in accordance with the manufacturer's protocol.

Adenovirus infection, cobalt treatment and immunoprecipitation of HUVEC. HUVEC were cultured to 90% confluency in a 6-cm plate. For infection, the growth medium was removed and 100 μl of adenovirus solution was added to the plate. One hour after incubation, 5 ml of the growth medium was added to the plate. Infected HUVEC were cultured for 36 h and treated with various concentrations of $CoCl_2$ for 4 h. After cobalt treatment, cells were harvested and lysed in 1 ml of lysis buffer (20 mM Tris–HCl (pH 7.6), 100 mM NaCl, 0.5% Igepal CA-630, 5 mM $MgCl_2$, 1 mM sodium orthovanadate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin A). After clearance by centrifugation, the supernatant was incubated for 2 h at 4°C with 40 μl of anti-FLAG M2 beads (Sigma). The beads were washed three times with Tris-buffered saline (TBS) (50 mM Tris–HCl (pH 7.5), 100 mM NaCl) and treated at 45°C for 1 h in 40 μl of sample buffer containing 2% SDS and 5% β-mercaptoethanol. The solubilized proteins were analyzed by Western blotting using rabbit anti-Cul2 antibody.

Fluorimetric detection of Co^{2+} binding. We examined the direct binding between GST-Cul2 and Co^{2+} using fluorescence spectroscopy. Since GST-Cul2$^{1–745}$ (full-length) was easily degraded in bacteria and had a low expression level, we used GST-Cul2$^{1–338}$ instead of GST-Cul2$^{1–745}$. As a negative control, plain GST was used. GST and GST-Cul2$^{1–338}$ were expressed in bacteria and purified as described above. Proteins were diluted to 1 μM in TBS. A cuvette was filled with 500 μl of the protein solution, and its fluorescence change was followed after each addition of 2.5 μl of $CoCl_2$ solution (100 μM in TBS). Inherent protein fluorescence quenching was monitored during titration by Co^{2+}. Fluorescence spectra were acquired on an SLM SPF 500C spectrofluorometer with an observation chamber equipped with a thermostat. The spectral band widths used were 280 nm for the excitation and 300 nm for the emission. Digitalized data were temporarily stored in the volatile memory of the built-in microprocessor in the fluorometer and then downloaded to an IBM-PC microcomputer for storage and further analysis. A standard Stern–Volmer plot was performed between F_0/F vs ion concentrations, when F_0 and F are the fluorescence in the absence and presence of metal ion, respectively. In addition to Co^{2+}, we examined the binding of GST or GST-Cul2$^{1–338}$ with various metal ions. We prepared 100 μM solutions of $CuCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $NiCl_2$ and added each one individually to protein solution.

RESULTS AND DISCUSSION

Binding of Cul2 to Co^{2+}-immobilized beads. To examine binding activity of Co^{2+} to Cul2, TALON beads were used. TALON beads were Co^{2+}-immobilized resin beads that were originally designed to purify recombinant proteins tagged with polyhistidine epitope such as 3RH (16). Cul2 and Uba3 (negative control) were tagged with 3HA or 3RH-epitope, overexpressed in COS cells, and precipitated by the Co^{2+} beads under denaturing condition. The precipitated proteins were detected by Western blotting using anti-HA antibody or anti-RH antibody. As shown in Fig. 1, both 3RH-Cul2 and 3RH-Uba3 were strongly expressed in total cell lysates (upper panel, lanes 2 and 3) and could be precipitated by Co^{2+} beads (upper panel, lanes 7 and 8). Both 3HA-Cul2 and 3HA-Uba3 were also strongly expressed in total cell lysates (middle panel, lanes 4 and 5). Interestingly, 3HA-Cul2 could be precipitated by Co^{2+} beads, whereas 3HA-Uba3 could not (middle panel, lanes 9 and 10). This result indicates that the Cul2 molecule itself has the ability to bind to Co^{2+} beads.

Cullin family members and their binding activity to Co^{2+}-immobilized beads. Cul2 belongs to the cullin family, which includes Cul1, Cul3, Cul4A, Cul4B, and Cul5. The sequence similarity has been shown to extend across all protein domains (17), suggesting that cullins other than Cul2 also bind to Co^{2+} beads. To examine this possibility, we used the same method as that performed in the experiment described above. As shown in Fig. 2, all cullins were expressed in COS cells (upper panel). However, only Cul2 (lane 3) and Cul4A (lane 5) were precipitated from the total cell lysate by Co^{2+} beads (middle panel). The precipitated amount of Cul2 was much higher than that of Cul4A, indicating Cul2′s higher binding affinity. The other cullins were not precipitated by Co^{2+} beads. Thus, among cullin family members, Co^{2+} beads bind specifically to Cul2. In addition to Co^{2+}, we examined the binding between Ni^{2+} and cullin family members because Ni^{2+} also stabilizes HIF1α (9). As shown in the lower panel, Ni^{2+} beads showed the same specificity as that of Co^{2+} beads.

Binding sites of Co^{2+}- or Ni^{2+}-immobilized beads in Cul2 molecule. Cul2 is composed of 745 amino acid residues with a calculated molecular mass of 87.0 kDa

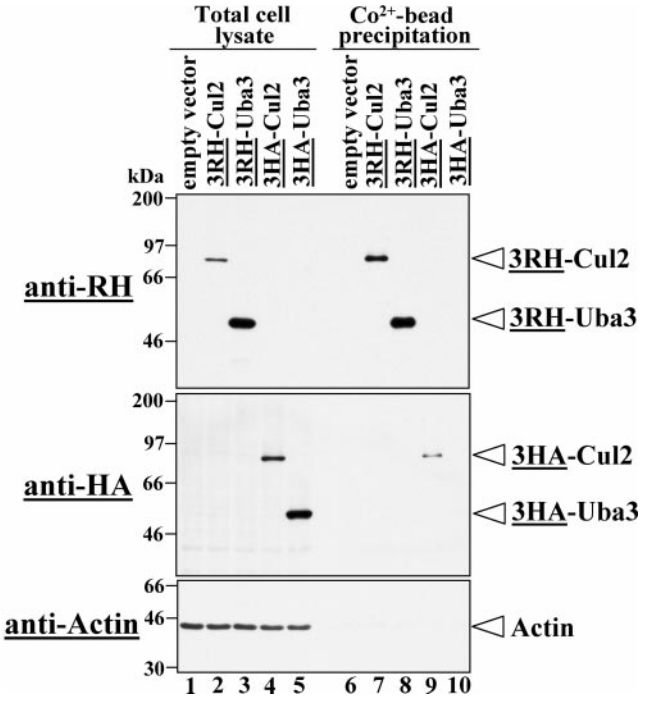

FIG. 1. Binding of Cul2 to Co^{2+}-immobilized beads. COS cells were transfected with plasmids to express empty vector (lanes 1 and 6), 3RH-Cul2 (lanes 2 and 7), 3RH-Uba3 (lanes 3 and 8), 3HA-Cul2 (lanes 4 and 9), or 3HA-Uba3 (lanes 5 and 10). Twenty hours after transfection, the total cell lysate was prepared from transfectants and analyzed by Western blotting (lanes 1–5). The total cell lysate was also used for TALON precipitation. The lysate was incubated with Co^{2+}-immobilized TALON beads for 1 h. After centrifugation, proteins bound to beads were analyzed by Western blotting (lanes 6–10). To detect 3RH-tagged proteins, anti-RH mAb was used (upper panel). To detect 3HA-tagged proteins, anti-HA mAb was used (middle panel). To demonstrate an equal loading amount of total cell lysate in each lane, anti-actin antibody was used (lower panel).

(GenBank Accession No. AF126404). Cul2 possesses several domains or motifs, including elongin C-binding site (amino acids 1–108) (18), NEDD8-conjugation site (Lys-689) (13), nuclear localizing signal (amino acids 676–693) (13, 17), and ROC1 binding site (19, 20). Because Cul2 was shown above to bind strongly to Co^{2+} and Ni^{2+} beads, we sought to define the domain in Cul2 that confers the binding activity. For this purpose, wild type and mutant Cul2 molecules were fused with GST at the amino terminus and expressed in bacteria. After lysis of the bacteria, the GST-fusion proteins were precipitated with the Co^{2+} or Ni^{2+} beads in 6 M guanidine HCl, washed in 8 M urea, and analyzed by Western blotting using anti-GST antibody (raw data not shown). As shown in Fig. 3, both Co^{2+} beads and Ni^{2+} beads precipitated GST-$Cul2^{1–745}$ (wild type), GST-$Cul2^{1–573}$, GST-$Cul2^{339–573}$, GST-$Cul2^{1–100}$, GST-$Cul2^{237–338}$, GST-$Cul2^{1–338}$, whereas the beads did not precipitate GST, GST-$Cul2^{101–236}$, or GST-$Cul2^{574–745}$. Thus, the binding sites of Co^{2+} and Ni^{2+} beads are localized in $Cul2^{1–100}$, $Cul2^{237–338}$, and $Cul2^{339–573}$ (Fig. 3), indicating that Cul2 has at least three binding sites.

Binding activity of Cul2 with divalent metal ions. We showed that Cul2 binds to Co^{2+}- or Ni^{2+}-immobilized beads. Although the binding between Cul2 and metal ion beads was clear, this result does not prove that direct binding occurs between Cul2 and Co^{2+}/Ni^{2+}. To determine whether direct binding occurs, we measured the quenching of intrinsic fluorescence of Cul2 upon Co^{2+} binding. As shown in Fig. 4 (upper panel), serial addition of Co^{2+} into GST-$Cul2^{1–338}$ caused specific large quenching of the protein's intrinsic fluorescence, indicating the direct binding of Co^{2+} to GST-$Cul2^{1–338}$. In contrast, other metal ions, namely, Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, resulted in only a small degree of nonspecific collision quenching, indicating minimal binding between these metal ions and GST-$Cul2^{1–338}$. Furthermore, GST did not bind to any of the common divalent cations, including Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (Fig. 4, lower panel). These results indicate that direct binding occurs between Co^{2+} and $Cul2^{1–338}$, leading to significant quenching of Cul2 fluorescence. In the separated experiments, we also examined the quenching of Cul2 fluorescence by Ni^{2+} using the same method and found the direct binding between Ni^{2+} and $Cul2^{1–338}$ (data not shown).

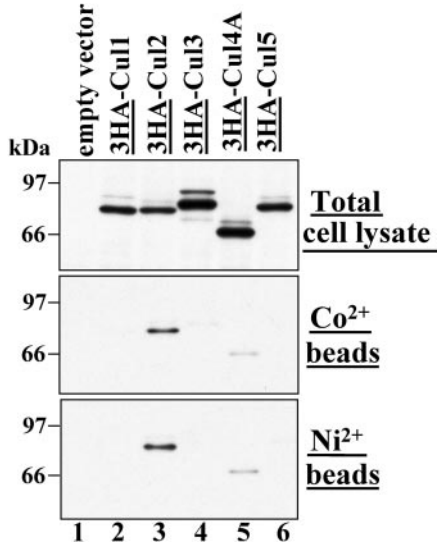

FIG. 2. Binding activity of cullin family members to Co^{2+}- or Ni^{2+}-immobilized beads. COS cells were transfected with plasmids to express empty vector (lane 1) or 3HA-tagged cullin family member (lanes 2–6). Twenty hours after transfection, the total cell lysate was prepared from transfectants and analyzed by Western blotting using anti-HA mAb (upper panel, lanes 1–6). The total cell lysate was also used for precipitation by Co^{2+}- (middle panel) or Ni^{2+}-immobilized beads (lower panel). The lysate was incubated with the beads for 1 h. After centrifugation, cullins bound to beads were detected by Western blotting using anti-HA mAb.

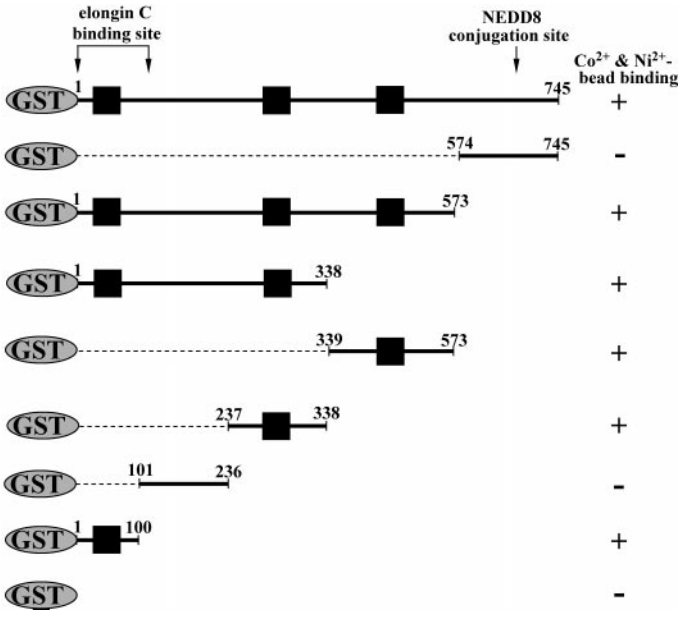

FIG. 3. Binding sites of Co^{2+} and Ni^{2+} beads in Cul2 molecule. Diagrammatic representation of the putative binding sites of Co^{2+} and Ni^{2+} beads in Cul2. The three binding motifs are located by using a spectrum of deletion mutants as indicated.

Binding of Cul2-associated molecules to Co^{2+} or Ni^{2+} beads. Cul2 is part of an SCF-like complex that contains elongin B, elongin C, and pVHL (21, 22). For the activation of this SCF-like complex, Cul2 has to be interacted with ROC1, and also modified by NEDD8. The activated SCF-like complex catalyzes ubiquitination of HIF1α and targets it for the proteosomal degradation (3, 4, 19, 23). We examined the binding activity of components of the SCF-like complex to Co^{2+} or Ni^{2+} beads. The component proteins were fused with GST at the amino terminus and expressed in bacteria. After lysis of the bacteria, the GST-fusion proteins were precipitated by Co^{2+} beads or Ni^{2+} beads under denaturing condition and analyzed by Western blotting using anti-GST antibody. Cul2 bound to metal ion beads, but other proteins, including pVHL, elongin B, elongin C, NEDD8, and ROC1, did not (data not shown). This result indicates that Cul2 is the only component of the SCF-like complex that can bind to Co^{2+} or Ni^{2+} beads.

Effect of Co^{2+} treatment on SCF-like complex formation in HUVEC. Cul2 binds to pVHL through elongins B and C and forms an SCF-like complex. We sought to examine whether the complex formation of Cul2 is affected by its binding with Co^{2+}. To do so, we had to determine the optimum condition for the treatment of HUVEC with $CoCl_2$. First, we examined the time course of HIF1α expression using HUVEC treated with 200 μM of $CoCl_2$ for various periods. HIF1α expression was not detected by 15 min after treatment, whereas it was detectable by 30 min and increased with time between 30 min and 2 h after treatment. There was no significant difference between 2 and 4 h after treatment (data not shown). We then examined the dose dependence of HIF1α expression by treating HUVEC with various concentrations of $CoCl_2$ for 4 h. As shown in Fig. 5A, HIF1α expression was not detected in HUVEC without $CoCl_2$ treatment (lane 1), but was increased, in a dose-dependent manner, in HUVEC treated with 50–200 μM $CoCl_2$ (lanes 2–4). There was no significant difference in HIF1α expression among populations of HUVEC treated with 200, 400, or 1000 μM $CoCl_2$ (lanes 4–6). Taken together, these results confirm that HIF1α is efficiently stabilized in HUVEC by treatment with $CoCl_2$ at 200–1000 μM for 2–4 h. Finally, we examined the effect of Co^{2+} treatment on formation of SCF-like-complex in HUVEC. The cells were infected with an adenovirus for the expression of FLAG-tagged pVHL and treated for 4 h with $CoCl_2$ at various concentrations. The cells were harvested and the total cell lysates were pre-

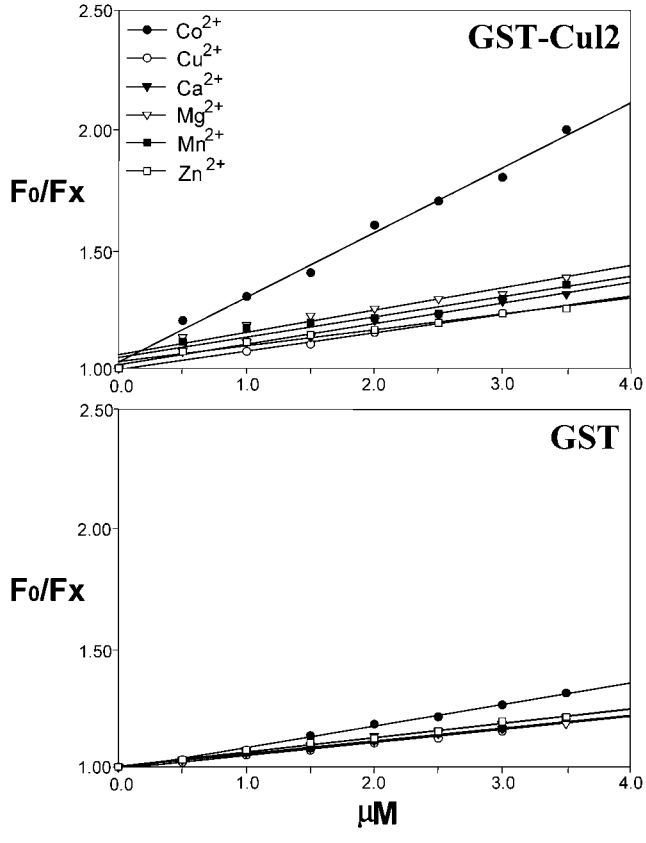

FIG. 4. Binding between Cul2 and divalent metal ions. We used fluorescence spectroscopy to show the direct binding of GST-$Cul2^{1-338}$ (upper panel) or GST (lower panel) with various metal ions, namely, Co^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. GST-$Cul2^{1-338}$ and GST were expressed in bacteria and purified. The protein solution was prepared at 1 μM in TBS and titrated with aliquots of each metal ion. Fluorescence spectra were acquired after each addition of metal titrant, and the fluorescence was calculated for Stern–Volmer analysis.

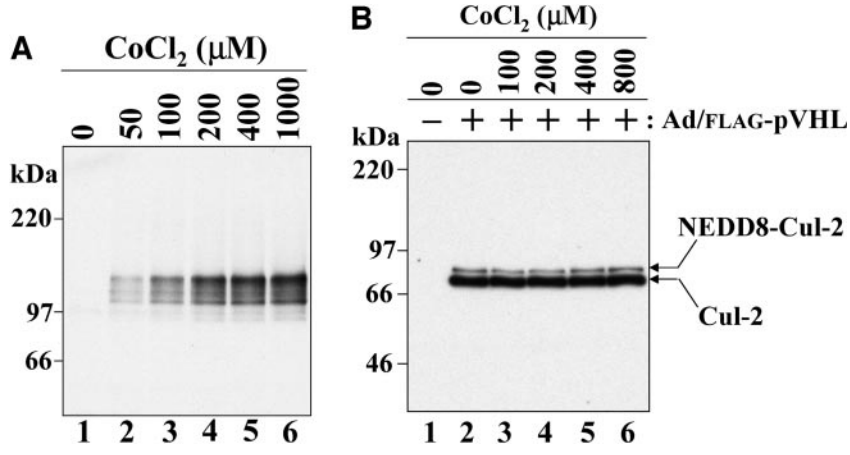

FIG. 5. Effect of Co^{2+} treatment on formation of SCF-like complex in HUVEC. (A) Dose dependence for Co^{2+}-mediated stabilization of HIF1α protein in HUVEC. HUVEC were left untreated (lane 1) or were treated for 4 h with $CoCl_2$ at concentrations of 50 (lane 2), 100 (lane 3), 200 (lane 4), 400 (lane 5), or 1000 μM (lane 6). After the treatment, total cell lysates were prepared and analyzed by Western blotting using anti-HIF1α mAb. (B) Effect of Co^{2+} treatment on the formation of SCF-like complex in HUVEC. HUVEC were left noninfected (lane 1) or were infected (lanes 2–6) with an adenovirus to express FLAG-pVHL. Thirty-six hours after infection, the cells were left untreated (lanes 1 and 2) or were treated for 4 h with $CoCl_2$ at concentrations of 100 (lane 3), 200 (lane 4), 400 (lane 5), or 800 μM (lane 6). Total cell lysates were prepared, and immunoprecipitation was performed using anti-FLAG mAb to precipitate an SCF-like complex composed of FLAG-VHL, elongin B, elongin C, Cul2, NEDD8, and ROC1. The coprecipitated Cul2 was detected by Western blotting using anti-Cul2 antibody.

pared. Then, immunoprecipitation was performed using an anti-FLAG mAb to precipitate an SCF-like complex composed of FLAG-pVHL, elongin B, elongin C, Cul2, NEDD8, and ROC1. The coprecipitated Cul2 was detected by Western blotting using anti-Cul2 antibody. As shown in Fig. 5B, the same amount of the unconjugated form and NEDD8-conjugated form of Cul2 could be coprecipitated from HUVEC treated with $CoCl_2$ at various concentrations (0–800 μM; lanes 2–6). Furthermore, we examined ROC1 in the coprecipitated samples. The same amount of ROC1 could be detected in the precipitates (data not shown). These results indicate that treatment with $CoCl_2$ shows no effect on the formation of SCF-like complex in HUVEC. In this study, we did not examine the effect of Ni^{2+} on the formation of SCF-like complex. Since Ni^{2+} has the binding activity as well as Co^{2+}, Ni^{2+} most likely shows no effect on the formation of SCF-like complex.

HIF1α plays a critical role in physiological responses to hypoxia and concerns with the transcriptional activation of many important genes such as VEGF and Glut1. It is also well known that hypoxia or treatment with divalent metal ions such as Co^{2+} and Ni^{2+} induces stabilization of HIF1α in mammalian cells. Recent studies have shown that HIF1α is modified through enzymatic hydroxylation of Pro564 and then recognized by pVHL, a part of E3ubiquitin ligase complex, resulting in the ubiquitination of HIF1α. Because the hydroxylation requires oxygen, this protein modification seems to be a key event in stabilization of HIF1α induced by hypoxic condition. However, the hydroxylation does not completely explain the mechanism of HIF1α stabilization induced by treatment with Co^{2+} or Ni^{2+}. In this study, we demonstrated three new findings. One is the direct binding of Co^{2+} and Ni^{2+} to Cul2. The second is that these metal ions do not bind to any components of the E3 ligase other than Cul2, suggesting that Cul2 is a key target of Co^{2+} and Ni^{2+}. The third is that Co^{2+} does not affect the complex formation of the E3 ligase for ubiquitination of HIF1α. However, Co^{2+} affects the degradation of HIF1α *in vivo*, implying that the metal binding to Cul2 affects the function, but not the assembly of the E3-ligase complex.

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