Cell, Vol. 111, 709–720, November 27, 2002, Copyright ©2002 by Cell Press

Regulation and Destabilization of HIF-1α by ARD1-Mediated Acetylation

Joo-Won Jeong,[1,2] Moon-Kyoung Bae,[1,2] Mee-Young Ahn,[2] Se-Hee Kim,[1] Tae-Kwon Sohn,[1,2] Myung-Ho Bae,[1,2] Mi-Ae Yoo,[2] Eun Joo Song,[3] Kong-Joo Lee,[3] and Kyu-Won Kim[1,4]

[1]Research Institute of Pharmaceutical Sciences
College of Pharmacy
Seoul National University
Seoul 151-742
Korea

[2]Department of Molecular Biology
Pusan National University
Busan 609-735
Korea

[3]Center for Cell Signaling Research
Division of Molecular Life Sciences and
College of Pharmacy
Ewha Womans University
Seoul 120-750
Korea

Summary

Hypoxia-inducible factor 1 (HIF-1) plays a central role in cellular adaptation to changes in oxygen availability. Recently, prolyl hydroxylation was identified as a key regulatory event that targets the HIF-1α subunit for proteasomal degradation via the pVHL ubiquitination complex. In this report, we reveal an important function for ARD1 in mammalian cells as a protein acetyltransferase by direct binding to HIF-1α to regulate its stability. We present further evidence showing that ARD1-mediated acetylation enhances interaction of HIF-1α with pVHL and HIF-1α ubiquitination, suggesting that the acetylation of HIF-1α by ARD1 is critical to proteasomal degradation. Therefore, we have concluded that the role of ARD1 in the acetylation of HIF-1α provides a key regulatory mechanism underlying HIF-1α stability.

Introduction

Eukaryotic cells sense oxygen and adapt to hypoxia by regulating a number of genes. In mammalian cells, the transcriptional complex HIF-1 plays an essential role in cellular and systemic oxygen homeostasis (Iyer et al., 1998; Semenza, 2000). HIF-1 stimulates the transcription of genes, such as erythropoietin and VEGF, whose protein products function to either increase oxygen availability by promoting erythropoiesis and angiogenesis, or activates genes involved in glucose transport and metabolism (Semenza, 1999).

HIF-1 is composed of two subunits: the hypoxia-regulated α subunit, HIF-1α, and the oxygen-insensitive HIF-1β subunit (Wang et al., 1995). Under normoxia, the HIF-1α subunit is rapidly degraded via the von Hippel-Lindau tumor suppressor gene product (pVHL)-mediated ubiquitin-proteasome pathway (Salceda and Caro, 1997; Huang et al., 1998; Maxwell et al., 1999; Berra et al., 2001). The association of pVHL and HIF-1α under normoxic conditions is triggered by the posttranslational hydroxylation of prolines (Pro402, Pro564) within a polypeptide segment known as the oxygen-dependent degradation (ODD) domain (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001) through the function of specific HIF-prolyl hydroxylases (HIF-PHs) (Bruick and McKnight, 2001; Epstein et al., 2001).

Reports show that p53 directly interacts with HIF-1α and limits hypoxia-induced expression of HIF-1α by promoting Mdm2-mediated ubiquitination and proteasomal degradation under hypoxic conditions (Ohh et al., 2000; Ravi et al., 2000). Furthermore, the degradation of HIF-1α by p53 in a hypoxic condition is inhibited by direct interaction with the Jun activation domain binding protein-1 (Jab1) and the ODD domain by blocking the interaction with p53 (Bae et al., 2002).

Phosphorylation and dephosphorylation in the signaling pathway can result in HIF-1 activation (Richard et al., 1999). During hypoxia, p42/p44 MAPK activity induces posttranslational phosphorylation of HIF-1α and promotes the transcriptional activity of HIF-1 (Richard et al., 1999). According to a recent report, the hydroxylation of asparagine (Asn803), located in the C-terminal transactivation domain (C-TAD) of HIF-1α, inhibits interaction with the p300/CBP coactivator and reduces the transcriptional activity of HIF-1 during normoxic conditions (Hewitson et al., 2002; Lando et al., 2002a, 2002b).

Thus, the modulation of HIF-1α stability and its activation involve multiple proteins and several posttranslational modifications. Until recently, studies of posttranslational modifications of HIF-1α were restricted to hydroxylation, ubiquitination, and phosphorylation. Other posttranslational modifications of HIF-1α have not been defined.

In the present study, we used the yeast two-hybrid system to identify proteins that interact with the ODD domain of HIF-1. One of the HIF-1α-interacting clones, mouse ARD1, shares 57% identity with the ARD1 acetyltransferase sequence of *Saccharomyces cerevisiae* (Tribioli et al., 1994). ARD1 is required for the expression of protein N-acetyltransferase (NAT) activity in lower eukaryotes and bacteria (Tribioli et al., 1994; Park and Szostal, 1992; Ingram et al., 2000). However, its function in mammalian cells has not been defined.

Acetylation is a posttranslational modification of proteins, with histones the best-known example (Kouzarides, 2000). Several enzymes are reported as histone acetyltransferases; these include PCAF, p300/CBP, SRC1, and MOZ (Kouzarides, 1999). Recently, these acetyltransferases have been shown to modify other proteins besides histones, including general transcription factors, e.g., E2F1, MyoD, GATA-1, and p53 (Kouzarides, 2000; Ogryzko et al., 1998; Boyes et al., 1998). Similar to phosphorylation, the posttranslational modification of general transcription factors by acetylation can regulate their DNA binding activity and interaction with other proteins such as coactivator proteins and tran-

[4]Correspondence: qwonkim@plaza.snu.ac.kr

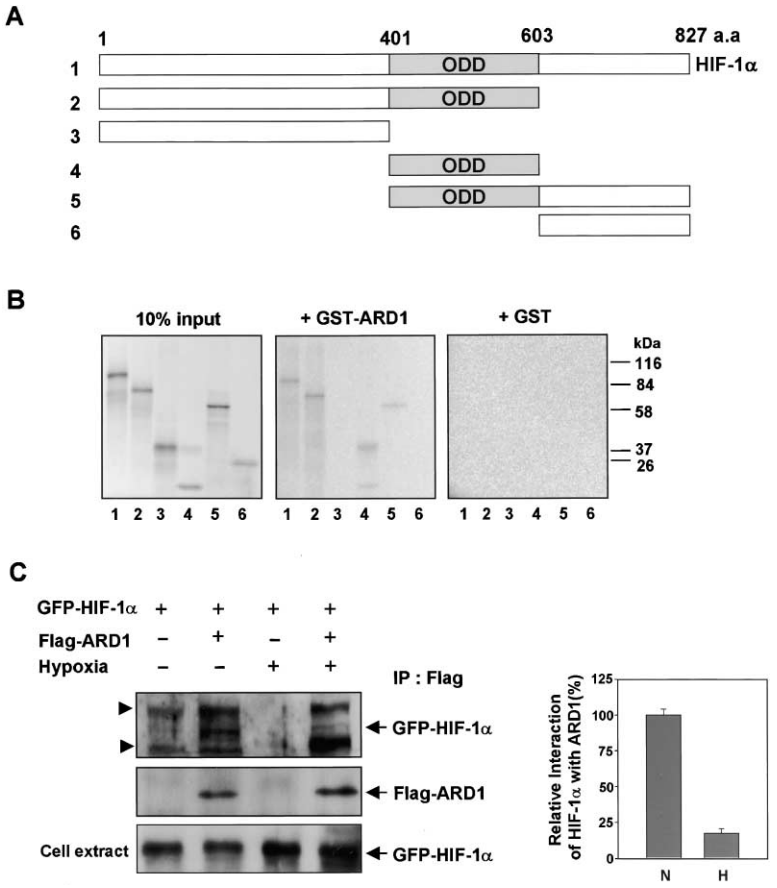

Figure 1. ARD1 Interacts with HIF-1α In Vitro and In Vivo

(A) Structures of HIF-1α and its derivatives are schematically shown. Gray boxes represent the ODD domain. 1, Full-length HIF-1α, 1–827 aa ; 2, 1–603 aa; 3, 1–400 aa; 4, 401–603 aa; 5, 401–827 aa; and 6, 604–827 aa.

(B) HIF-1α and its deletion derivatives were translated by programmed reticulocyte lysate in the presence of [^{35}S]methionine. 10% of the material used in the GST pull-down experiment is shown (left). In vitro translated proteins were mixed with GST-ARD1 bound beads (middle) or GST bound beads (right), and a GST pull-down assay was performed. The numbers at the bottom indicate the derivatives of HIF-1α shown in (A).

(C) HEK293 cells were transfected with expression vector for CMV-ARD1 and/or GFP-HIF-1α as indicated. After 24 hr posttransfection, the cells were incubated under hypoxia and the cell extracts were prepared. Total cell lysates were immunoprecipitated with anti-FLAG antibody. The presence of GFP-HIF-1α in the immunoprecipitates was examined using anti-HIF-1α antibody (top). Arrowheads at left indicate nonspecific bands. Immunoprecipitated materials and total cell extracts were analyzed by Western blots with anti-Flag antibody and anti-HIF-1α antibody, respectively (middle, bottom). The relative interactions from three independent assays were quantified by densitometry. The interaction of ARD1 with HIF-1α under normoxia was set to 100% (right). IP, immunoprecipitation; N, normoxia, 21% O_2; H, hypoxia, 1% O_2.

scriptional regulator proteins (Bannister and Miska, 2000; Barlev et al., 2001). However, studies of protein acetyltransferases, except for histone acetyltransferases, and their substrates are in an early stage.

In this report, we established the function of ARD1 as a protein acetyltransferase in mammalian cells, with HIF-1α a possible target protein for the enzyme. Moreover, we show that the acetylation of HIF-1α by ARD1 regulates the stability of HIF-1α in response to O_2 concentration and further demonstrate that the acetylation of HIF-1α by ARD1 can be coordinated with other posttranslational modifications, prolyl hydroxylation, and ubiquitination.

Results

Identification of ARD1 for HIF-1α-Interacting Protein

We used the yeast two-hybrid system to identify candidate proteins that interact with HIF-1α in vivo. To perform the two-hybrid assay, the *his*$^-$ yeast strain HF7C was cotransformed with a bait vector, encoding the GAL4 DNA binding domain in-frame with HIF-1α residues 401–603 (ODD domain) (Huang et al., 1998) and prey vectors, allowing expression of mouse embryonic and T cell lymphoma cDNA sequences in-frame with GAL4 transactivation domain. The bait did not show any self-activity of transcriptional activation for the reporters. We screened 6×10^6 yeast transformants for histidine auxotrophy and β-galactosidase expression. From these transformants, 190 His$^+$/Lac$^+$ double-positive clones were isolated and 64 clones were further selected by β-galactosidase assays in another yeast strain, SFY 526, containing a GAL1UAS-*Lac*Z reporter gene.

DNA sequencing and database searches revealed that the nucleotide sequences of 13 clones (5 clones from a 17 day embryonic mouse cDNA library and 8 clones from mouse T cell cDNA library) correspond to a 700 bp mouse cDNA of a mouse homolog for ARD1 N-acetyltransferase (GenBank accession number NM_019870). This gene, called mouse ARD1 in GenBank databases, has 96% homology to the human homolog ARDH (NM_003491) and 57% homology to *S. cerevisiae* ARD1 (M11621).

Direct interaction of ARD1 with HIF-1α in vitro was examined using GST pull-down assays. We synthesized ^{35}S-labeled deletion constructs of HIF-1α (Figure 1A) in vitro, then tested for interactions with bacterially expressed glutathione-S-transferase (GST) or GST-fused ARD1. The results shown in Figure 1B indicated that three deletion constructs, including the ODD domain, (HIF-1α 1–603, 401–603, 401–827) and the full-length HIF-1α were pulled down by interaction with the GST-fused ARD1 protein, whereas the amino acid 1–400 and 604–827 regions of HIF-1α showed no interactions. None of the HIF-1α constructs used in this assay bound GST alone.

Interaction between ARD1 and HIF-1α in vivo was confirmed by coimmunoprecipitation assays. To overexpress the ARD1 and HIF-1α proteins in HEK 293 cells,

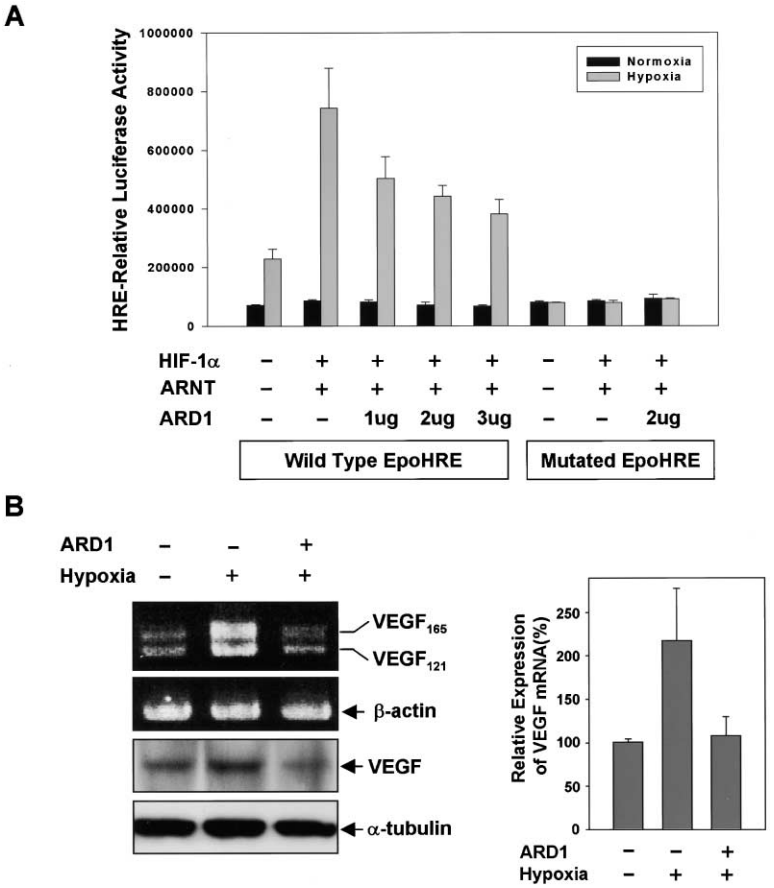

Figure 2. ARD1 Decreases the HIF-1α-Mediated Transactivation Function and VEGF Expression

(A) HEK293 cells were cotransfected with pSV40pro-EpoHRE-Luc (1 μg) or mutated EpoHRE-Luc (1 μg), pBOS-hHIF-1α (0.1 μg), pBOS-hARNT (0.1 μg), or pEF-BOS alone (0.2 μg) and 2 μg of pCMV-ARD1 or pCMV alone as indicated. Transfected cells were incubated for 24 hr at 21% O_2 and then incubated at 21% O_2 or 1% O_2 for an additional 16 hr. The mean and standard deviation based on three (mutated EpoHRE) or six (wild-type EpoHRE) independent transfections are shown.

(B) HT1080 cells were stably transfected with the indicated expression vector and left untreated or exposed to 1% O_2 for 16 hr. RT-PCR analysis (top two) was performed to detect gene expression using specific primer for VEGF and β-actin. Anti-VEGF and anti-α-tubulin Western blots from total protein extracts (bottom two) were performed. The VEGF mRNA expression level was measured in four independent experiments followed by densitometry. The expression under normoxia was set to 100% (right).

we cotransfected the Flag-tagged ARD1 expression vector and the GFP-HIF-1α expression vector into HEK 293 cells. When the Flag-ARD1 protein was immunoprecipitated from transfected cell extracts using the anti-α-Flag antibody, the GFP-HIF-1α protein was coimmunoprecipitated (Figure 1C). ARD1 interacted with HIF-1α under both normoxic and hypoxic conditions; however, this interaction was decreased when cells were exposed to 1% O_2, in spite of the higher GFP-HIF-1α expression level under this condition (Figure 1C).

ARD1 Inhibits Transcriptional Activation Mediated by HIF-1

To investigate whether ARD1 protein is involved in HIF-1 transcriptional activity, we used a luciferase reporter system, pSV40promoter-EpoHRE-Luc, and mutated EpoHRE-Luc reporters. Upon transient transfection of HIF-1α, ARNT, and EpoHRE-Luc vectors, reporter activity was markedly increased in cells exposed to 1% O_2 (Figure 2A). However, the overexpression of ARD1 was shown to decrease the transcriptional activity of HIF-1 in a dose-dependent manner. In cells transfected with the mutated EpoHRE reporter, luciferase activity was not changed by either hypoxia or the ARD1 protein.

Furthermore, to evaluate whether ARD1 modulates the hypoxia-response element (HRE)-containing VEGF gene, HT1080 cells were stably transfected with the Flag-tagged ARD1 expression vector, pCMV-ARD1. HT1080 cells overexpressing the ARD1 protein were designated as HT1080-ARD1 cells. As shown in Figure 2B, the increased expressions of VEGF mRNA and protein by hypoxia were downregulated in HT1080-ARD1 cells. These results suggest that the ARD1 protein negatively regulates the hypoxia-inducible transcriptional activity of HIF-1.

ARD1 Downregulates HIF-1α Protein Stability

Since the ARD1 protein directly binds to the ODD domain, we examined the effect of ARD1 on HIF-1α protein stability. As shown in Figure 3A, the overexpressed ARD1 downregulated the protein level of HIF-1α induced by hypoxia, but it did not affect mRNA level of HIF-1α, suggesting that the reduction of the HIF-1α protein level by ARD1 was not due to the transcriptional reduction of the HIF-1α gene. This suggests that the stability of the HIF-1α protein is reduced by ARD1 protein.

To confirm this notion, we added MG132, a specific inhibitor of the 26S proteasome, to HT1080-ARD1 cells and checked the level of HIF-1α protein. In the presence of MG132, the HIF-1α protein level decreased by ARD1 was restored to the level of hypoxia-treated HT1080 cells (Figure 3B). Furthermore, in pulse-chase experiments, the half-life of the HIF-1α protein was decreased in HT1080-ARD1 cells compared to HT1080 cells, thereby confirming the effect of ARD1 on the downregulation of HIF-1α stability (Figure 3C).

The downregulation of the HIF-1α protein level by ARD1 under normoxic conditions was undetectable be-

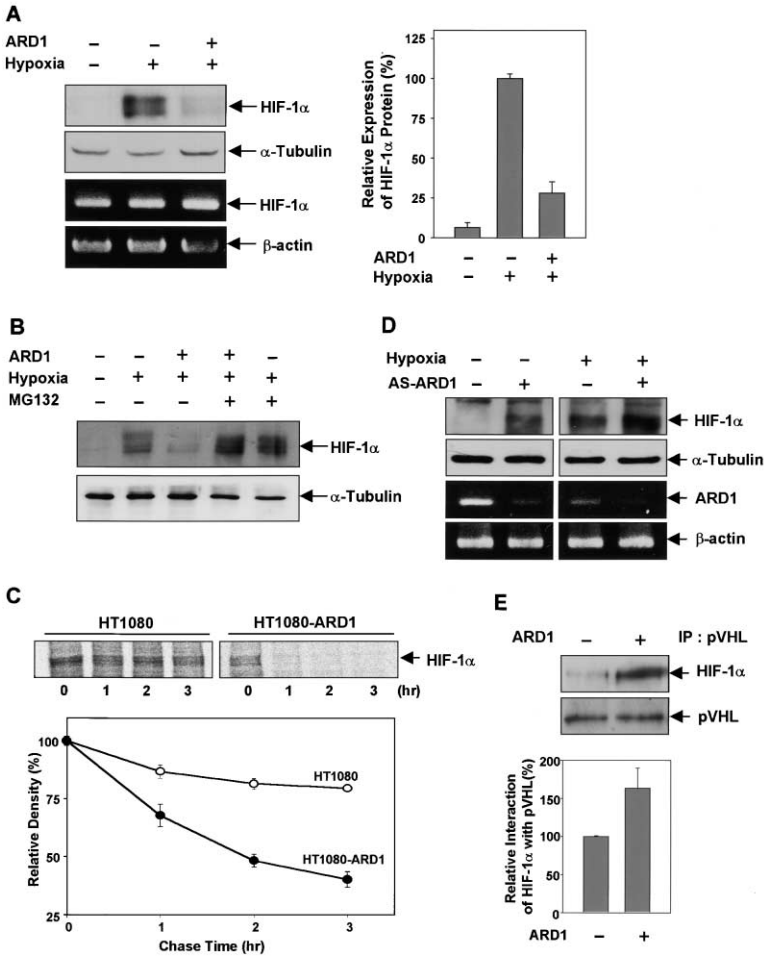

Figure 3. ARD1 Decreases HIF-1α Stability and Regulates Association between HIF-1α and pVHL

(A) HT1080 and HT1080-ARD1 cells were exposed to 1% or 21% O_2 for 2 hr. HIF-1α protein levels and mRNA levels were examined by Western blot analysis (top two) and RT-PCR analysis (bottom two), respectively. The relative HIF-1α protein level from six independent assays was quantified by densitometry. The HIF-1α protein level under hypoxia was set to 100% (right).

(B) HIF-1α protein in HT1080 and HT1080-ARD1 cells exposed to 1% O_2 for 2 hr followed by the addition of 5 μM MG132 for 1 hr as indicated. Cell lysates were harvested and subjected to immunoblot analysis of HIF-1α.

(C) HT1080 (open circles) and HT1080-ARD1 (closed circles) cells were pulse labeled with [^{35}S]methionine and chased in unlabeled medium for the indicated times under hypoxia. Cell lysates were immunoprecipitated with anti-HIF-1α antibody. The intensities of HIF-1α signals from three independent experiments were quantified and plotted.

(D) HEK293 cells were transfected with the antisense ARD1 expression vector and left untreated or exposed to 1% O_2 for 2 hr. HIF-1α protein levels by Western blot were examined (top two) and diminished ARD1 mRNA by the antisense ARD1 expression vector was checked using RT-PCR (bottom two).

(E) Endogenous pVHL was immunoprecipitated from 5 μM MG132 treated HT1080 and HT1080-ARD1 cell extracts with anti-pVHL antibody, and coprecipitated HIF-1α was detected by Western blot analysis. Immunoprecipitated pVHL was visualized by Western blot analysis with anti-pVHL antibody for loading control (bottom). The relative interaction from three independent experiments was quantified by densitometry. The interaction of HIF-1α with pVHL in HT1080 cells was set to 100% (bottom).

cause HIF-1α is not present in this condition. However, HIF-1α stability under normoxia was significantly increased by transfection of the antisense ARD1 expression vector (Figure 3D). These data strongly suggest that endogenous ARD1 induces the degradation of HIF-1α under normoxia. Similarly, HIF-1α stability under hypoxia was increased by the antisense ARD1 expression vector.

In addition, we investigated whether ARD1 participates in the association of HIF-1α and pVHL, which negatively regulates HIF-1α stability. We found that under normoxic conditions, ARD1 overexpression increased interaction of HIF-1α with pVHL (Figure 3E). These results suggest that ARD1 could function as a negative regulator of HIF-1α stability by accelerating the recruitment of pVHL to HIF-1α.

ARD1 Acetylates the ODD Domain of HIF-1α

The function of the ARD1 protein as an acetyltransferase is known in lower eukaryotes, such as *S. cerevisiae*; however, the function and the substrates of ARD1 in mammalian cells have not been identified. To determine if HIF-1α can be acetylated by the acetyltransferase activity of ARD1, we first assessed whether stimulated acetylation affects the protein level of HIF-1α. To do that, we treated HT1080 cells with sodium butyrate, an inhibitor of protein deacetylases. As shown in Figure 4A, sodium butyrate dramatically decreased the hypoxia-induced HIF-1α protein level. A histone deacetylase inhibitor, TSA, also decreased the expression of HIF-1α protein (Figure 4A). These results suggest that the degradation of HIF-1α is stimulated by protein acetylation.

To obtain in vivo evidence showing the acetylation of HIF-1α protein, we transfected HIF-1α expression vector into HEK 293 cells and performed an in vivo acetylation assay using an anti-Ac-Lys antibody. The results shown in Figure 4B indicate that lysine residue(s) in ectopic HIF-1α was acetylated in vivo and that this acetylation was stimulated by the overexpression of the ARD1 protein.

Next, we examined the relative acetylated HIF-1α under normoxic and hypoxic conditions, because the rate of HIF-1α acetylation may be not equal under these conditions. Although tests revealed that the protein level of HIF-1α was almost the same under these two conditions when treated with MG132, the acetylation level of

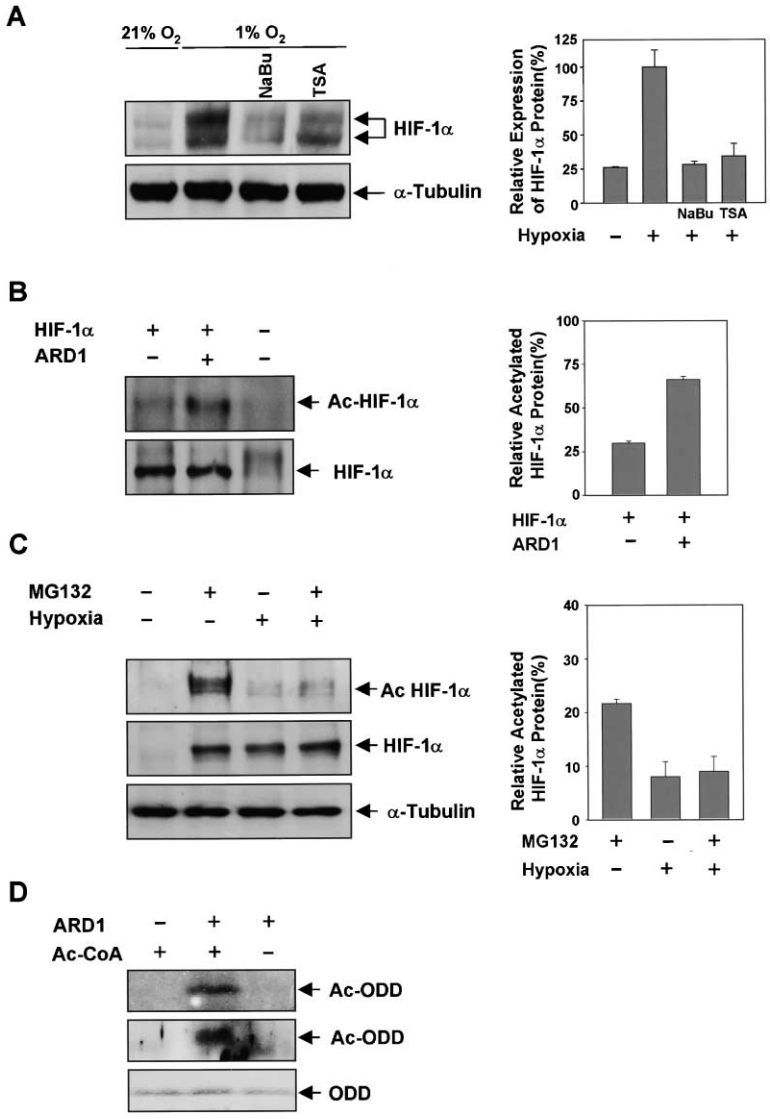

Figure 4. ARD1 Acetylated the ODD Domain of HIF-1α

(A) HT1080 cells were exposed to 1% O_2 for 2 hr with addition of 10 mM NaBu or 300 ng/ml TSA as indicated. Cell lysates were harvested and subjected to immunoblot analysis of HIF-1α. The relative HIF-1α protein level from four independent assays was quantified by densitometry. The HIF-1α protein level under hypoxia was set to 100% (right).

(B) For ectopic expression of HIF-1α, HEK293 cells were transfected with pBOS-hHIF-1α expression vector. Extracts were immunoprecipitated with anti-Ac-Lys antibody, and immunoprecipitates were subjected to Western blot analysis for HIF-1α. The relative acetylated HIF-1α, the acetylated HIF-1α/expressed HIF-1α ratios, was normalized to the results of immunoprecipitation and Western blot analysis using densitometer from three independent assays (right).

(C) HT1080 cells were exposed to 5 μM MG132 or 1% O_2 for 2 hr as indicated and cell lysates were immunoprecipitated with anti-Ac-Lys antibody. Cell lysates and immunoprecipitates were subjected to Western blot analysis with anti-HIF-1α antibody. The relative acetylated HIF-1α, the acetylated HIF-1α/expressed HIF-1α ratios, was normalized to the results of immunoprecipitation and Western blot analysis from five independent assays (right).

(D) Following incubation of purified ODD domain (400 ng) with 50 nCi [^{14}C]acetyl-CoA (top) or 10 mM acetyl-CoA (middle) and 100 ng of purified ARD1 protein for 2 hr at 30°C, the reaction products were separated by SDS-PAGE and visualized by autoradiography (top) and Western blot analysis with anti-Ac-Lys antibody (middle). The input ODD domain proteins were shown using Coomassie brilliant blue staining (bottom).

HIF-1α in normoxia was much higher than that of HIF-1α in hypoxia or under a MG132-treated hypoxic condition (Figure 4C).

Our next question to answer was whether HIF-1α could serve as a direct substrate for ARD1 and whether ARD1 protein can function as a HIF-1α acetyltransferase by direct interaction with the ODD domain. To test these possibilities, we performed an in vitro acetyltransferase assay using purified recombinant ODD and ARD1 proteins. As shown in Figure 4D, lysine residues of the ODD domain were acetylated in the presence of ARD1 protein and Ac-CoA, whereas acetylated ODD was not detected in the absence of either ARD1 or Ac-CoA. These results strongly suggest that the ARD1 protein acetylates the ODD domain of HIF-1α by transferring acetyl group from Ac-CoA to lysine residues in the ODD domain through direct interaction.

ARD1 Acetylates the Lys532 in HIF-1α Protein

In general, the position-specific acetylation of a target protein is mediated by distinct acetyltransferases (Gu and Roeder, 1997). There are six lysine residues in the ODD domain of HIF-1α, namely K443, K460, K477, K532, K538, and K547. To identify the acetylated lysine(s) of the ODD domain by ARD1, we performed site-directed mutagenesis using pET-ODD expression vector to change the six single lysines to arginine residues. The six mutated ODD proteins were purified and an in vitro acetyltransferase assay was executed. Interestingly, as shown in Figure 5A, ODD-K532R was not acetylated by ARD1. However, the other mutated ODD proteins (ODD-K443R, -K460R, -K477R, -K538R, and -K547R) were acetylated to a similar level as the wild-type ODD protein. Therefore, we suggest that ARD1 protein specifically acetylates Lys532 in the ODD domain of HIF-1α.

To confirm the acetylation site of the ODD domain, the in vitro acetylated ODD protein by ARD1 protein was purified by immunoprecipitation with Ac-Lys antibody and digested with V8 protease, and then the resulting peptides were analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The masses of these peptides were

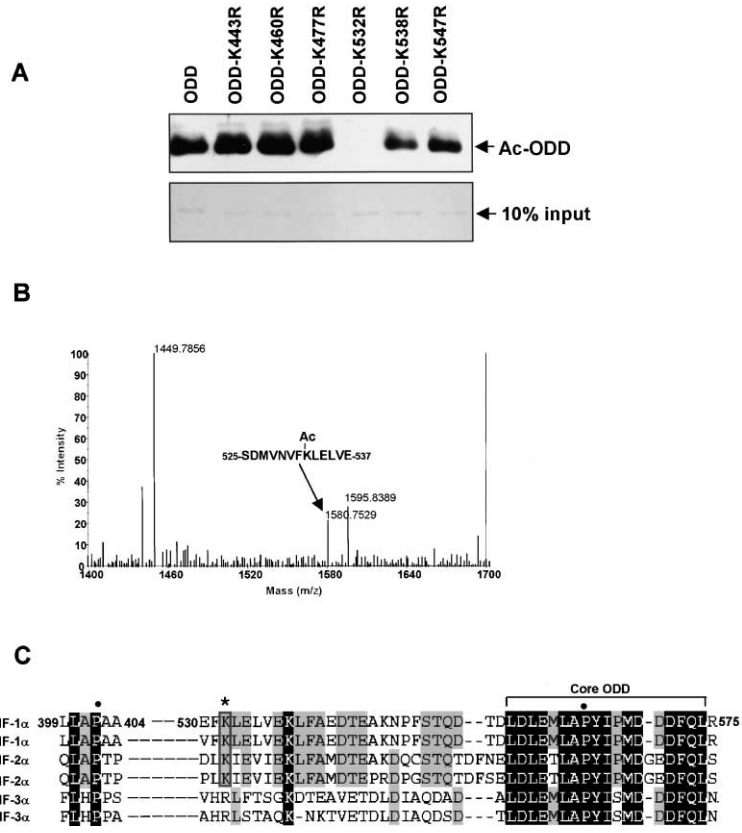

Figure 5. The Acetylation Site of HIF-1α by ARD1 Is Lys532 in ODD Domain

(A) Purified recombinant ODD and mutated ODD domain proteins were incubated with acetyl-CoA and ARD1 protein for acetylation reaction. In vitro acetylated proteins were subjected to Western blot analysis with anti-Ac-Lys antibody. 10% of the materials used in this assay was shown by staining with Coomassie brilliant blue (bottom).

(B) In vitro acetylated ODD protein with acetyl-CoA and ARD1 was purified using anti-Ac-Lys antibody and cleaved with V8 protease, and then was subjected to MALDI-TOF MS. The arrow indicated the acetylated peptide peak.

(C) Sequence alignments of human and mouse HIF-α proteins. The asterisk denotes the lysine residue that acts as a target for ARD1-induced acetylation. Two dots denote the conserved prolines (Pro402 and Pro564) for hydroxylation.

compared with masses of expected peptides calculated from the known sequence (Figure 5B). New peptide at m/z 1580.7529 Da was detected in the ODD domain acetylated by ARD1 protein. This is the expected mass of acetylated (42.0106 Da increase) ^{525}SDMVNVFKLELVE^{537} peptide containing oxidation of Met (15.9950 Da increase) (Figure 5B). This result confirmed the target site of ARD1 as Lys532 in HIF-1α.

It is worth noting that this lysine residue is conserved in HIF-1α and -2α, which are oxygen sensitive, whereas HIF-3α, stable even in normoxia (Hara et al., 2001), does not contain this residue (Figure 5C). However, two proline residues for hydroxylation are conserved in the three HIF-α proteins.

ARD1 as a HIF-1α Acetyltransferase Stimulates HIF-1α Degradation

As described above, Lys532 is the target site for acetylation by ARD1 protein, and ARD1 decreased the stability of HIF-1α. In a previous report, a Lys532-mutated N-terminal transactivation domain (N-TAD) of HIF-1α was not degraded under normoxic conditions (Tanimoto et al., 2000). The authors suggested that Lys532 is critical to the degradation of HIF-1α protein. Since Lys532 is the acetylation site, we suggest that the acetylation of Lys532 by ARD1 is actively involved in the degradation of HIF-1α under normoxic conditions.

To prove this point, we transfected the Flag-tagged HIF-1α-K532R expression vector into HEK 293 cells and checked the protein level under normoxic conditions. As shown in Figure 6A, the transfected HIF-1α-K532R protein was stable in normoxia, whereas wild-type HIF-1α was degraded under the same condition. To determine the half-life of the HIF-1α-K532R protein, we performed pulse-chase experiments using transiently transfected HEK293 cells under normoxia. The results confirmed that HIF-1α-K532R protein is more stable than wild-type HIF-1α protein under normoxia (Figure 6A). Moreover, the interaction of pVHL with HIF-1α-K532R protein was much weaker than that with wild-type protein (Figure 6B). These results suggested that HIF-1α-K532R protein is stable under normoxic conditions when pVHL interaction is reduced.

Based on the finding that the K532R mutant protein was not acetylated by ARD1, we expected that the acetylation of Lys532 in HIF-1α stimulates the degradation of this protein through increasing pVHL-mediated ubiquitination. To examine this possibility, we first performed a pull-down assay to test for the interaction between the ODD domain and pVHL. As shown in Figure 6C, ARD1-treated (acetylated) wild-type ODD protein increased the interaction with pVHL, whereas ARD1-treated ODD-K532R mutant protein did not.

To test whether acetylation by ARD1 can stimulate HIF-1α ubiquitination, we performed an in vitro ubiquitination assay using purified ODD protein. The result in Figure 6D indicates that acetylation by ARD1 increased ubiquitination of the ODD domain. Moreover, the ODD-K532R mutant protein was ubiquitinated at the same level as the wild-type, implying that Lys532 is not a ubiquitination site (Figure 6E). In addition, we observed

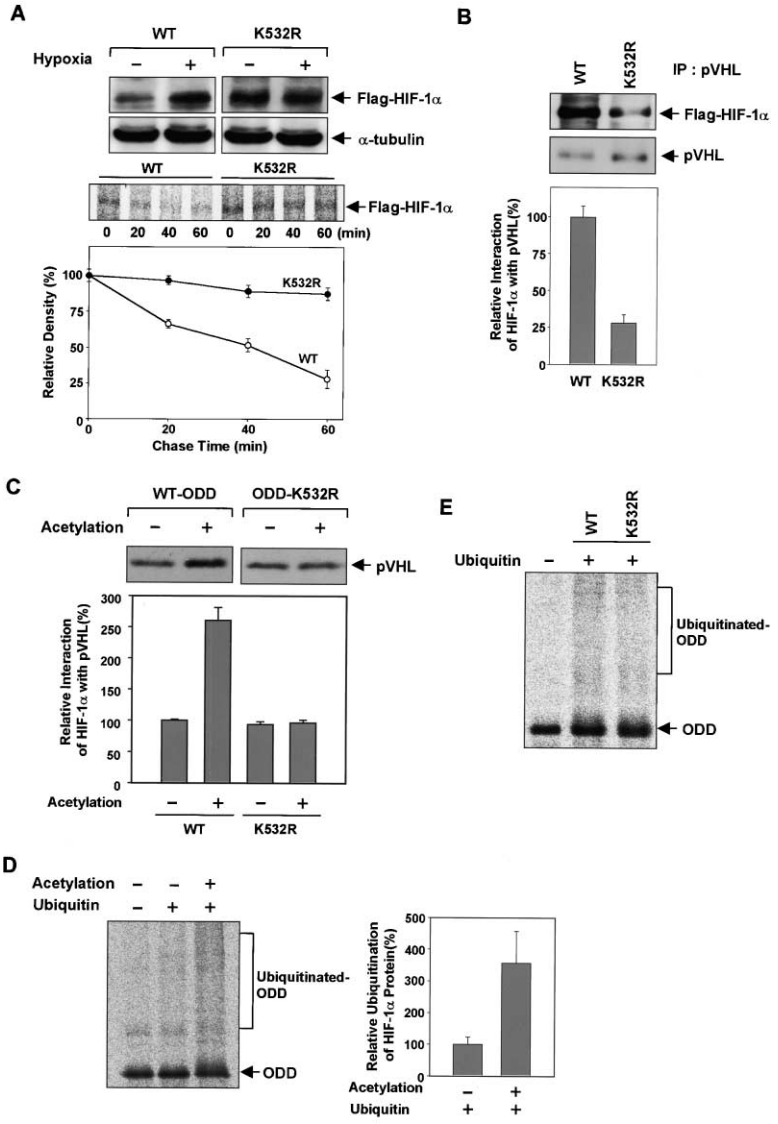

Figure 6. The HIF-1α-K532R Mutant Is Not Degraded under Normoxic Conditions

(A) Flag-tagged wild-type or mutant HIF-1α (substitution of Lys532 to Arg) was transiently expressed in HEK293 cells and incubated under normoxic or hypoxic conditions for 6 hr. Cell extracts were prepared and subjected to immunoblot analysis with anti-Flag antibody (top two). Wild-type (open circles) and K532R mutant (closed circles) HIF-1α-transfected HEK293 cells were pulse labeled with [^{35}S]methionine and chased in an unlabeled medium for the indicated times under normoxia. Cell lysates were immunoprecipitated with anti-Flag antibody. The intensities of Flag-HIF-1α signals from three independent experiments were quantified and plotted.

(B) After transfection of HEK293 cells with the indicated vectors, 5 μM MG132 was added for 2 hr and total cell extracts were isolated. Endogenous pVHL was immunoprecipitated and coprecipitated HIF-1α was detected by Western blot analysis. The relative interaction from three independent experiments was quantified by densitometry. The interaction of wild-type HIF-1α with pVHL was set to 100% (bottom).

(C) Purified His-ODD or His-ODD-K532R protein was acetylated by ARD1 as indicated and products were preincubated in the presence of HeLa cell extract at 30°C for 2 hr. Pull-down reaction was performed with ^{35}S-labeled pVHL for 1 hr and precipitants were subjected to SDS-PAGE and autoradiography. The interaction signals from three independent experiments were quantified (bottom).

(D) ^{35}S-labeled ODD was acetylated by ARD1 as indicated and the products were preincubated with HeLa cell extract. The in vitro ubiquitination assay was performed at 30°C for 2 hr and the reaction products were subjected to SDS-PAGE and autoradiography. Levels of total and ubiquitinated ODD in the second and third lanes from left were quantified by densitometery. The ratio of ubiquitinated/total ODD was determined and normalized to the result obtained in the second lane to yield the relative HIF-1α ubiquitination (right).

(E) ^{35}S-labeled ODD and ODD-K532R were preincubated with HeLa cell extract. The in vitro ubiquitination assay was performed at 30°C for 2 hr and the reaction products were subjected to SDS-PAGE and autoradiography.

the hydroxylation of Pro564 in the ODD domain under ARD1-mediated acetylation by the MALDI-TOF MS analysis (data not shown). These results indicate that the acetylation of Lys532 by ARD1 protein is a key regulatory event for the maximal degradation of HIF-1α, mediated by the pVHL ubiquitination complex under proline hydroxylated conditions.

ARD1 Expression Is Decreased under Hypoxic Conditions

The EST data from UniGene Cluster Mm. 5934 indicated that mouse ARD1 is expressed in almost every cell type. To confirm the expression of ARD1 mRNA, we performed Northern blot analysis of RNAs from various human tissues. As shown in Figure 7A, ARD1 was detected in all tissues tested, including brain, heart, skeletal muscle, and liver, suggesting that ARD1 is broadly expressed.

To examine the expression level of ARD1 during hypoxia, we performed reverse transcriptase PCR. The ARD1 mRNA level was decreased in cells exposed to 1% O_2 and was also decreased when cells were treated with hypoxia-mimicking agents, cobalt chloride and 2,2′-dipyridyl (Figure 7B).

Our next question was the relation between ARD1 mRNA reduction and HIF-1α stability. We therefore checked the mRNA level of ARD1 and the acetylation and protein levels of HIF-1α after hypoxic exposure for 2 and 4 hr. As shown in Figure 7C, ARD1 mRNA level decreased upon exposure to hypoxia for 2 and 4 hr. The acetylation of HIF-1α was also decreased in proportion to the increase of hypoxic exposure, whereas the protein level of HIF-1α was increased. These data support the

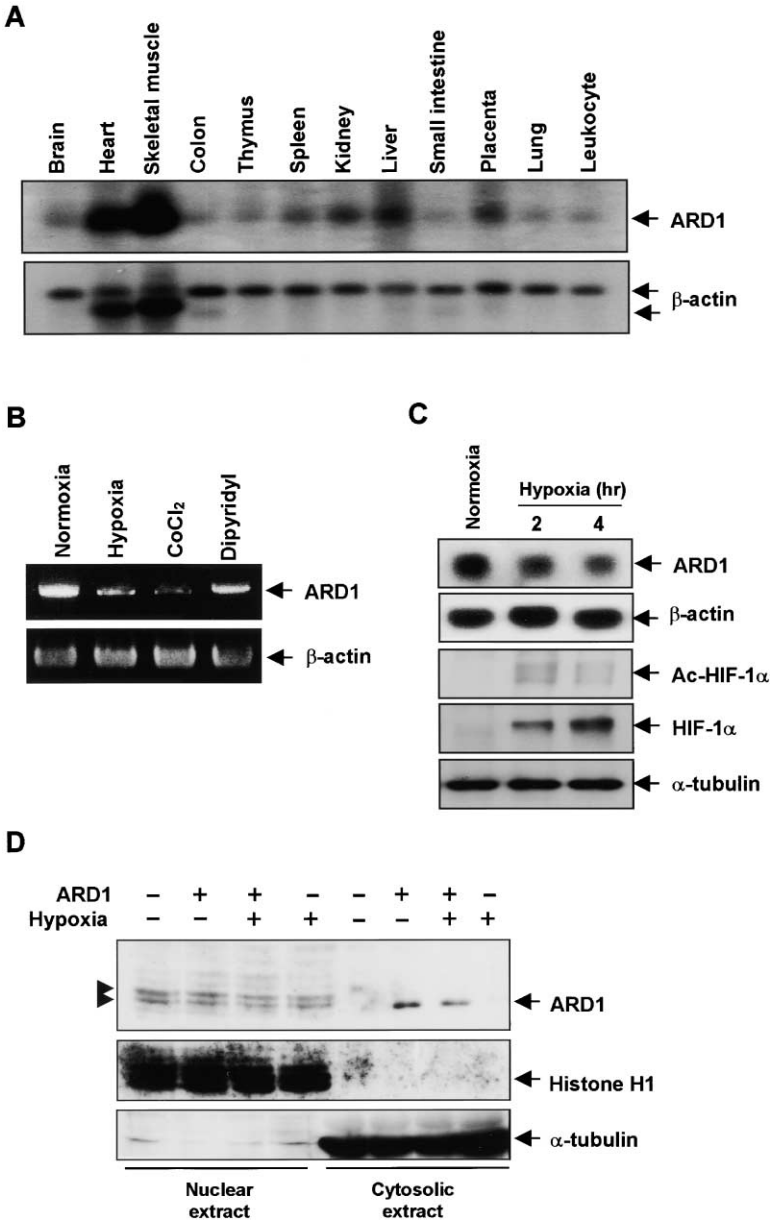

Figure 7. ARD1 Expression Is Decreased under Hypoxic Conditions

(A) The expression of hARD1 mRNA was detected by Northern blot analysis using human multiple-tissue blot. A human β-actin probe was used as a loading control.

(B) HT1080 cells were incubated for 2 hr under hypoxic conditions or treated with 100 μM cobalt chloride and 100 μM 2,2′-dipyridyl. RT-PCR analysis was performed using specific primers for ARD1 and β-actin.

(C) mRNA and cell lysates were isolated from HT1080 cells exposed to 1% O_2 for 2 and 4 hr, then Northern blot analysis using specific probe for ARD1 and β-actin (top two), acetylation assay for HIF-1α (middle), and Western blot analysis for HIF-1α and α-tubulin (bottom two) were performed.

(D) The nuclear and cytosolic extracts were prepared from HT1080 or HT1080-ARD1 cells exposed to 1% or 21% O_2 for 2 hr. Extracts were subjected to Western blot analysis with anti-Flag antibody. Arrowheads at left indicate nonspecific bands (top). Immunoblotting was used to confirm purity of nuclear and cytosolic extracts using antisera against histone H1 and α-tubulin, respectively.

hypothesis that decreased expression of ARD1 causes increased HIF-1α stability by reduction of acetylation under hypoxia.

To obtain information on the subcellular localization of ARD1 protein, we separately prepared nuclear extracts and cytosolic extracts of ARD1 transfected cells. ARD1 protein was detected only in the cytosolic fraction, not in nucleus (Figure 7D), indicating that ARD1 may act on HIF-1α in the cytosol.

Discussion

The regulation of HIF-1α stability and its activity occur at multiple levels. The modulation of HIF-1α protein by interaction with other proteins is known to play an important role in its stabilization and activation. Recent work with mammalian cells demonstrated that posttranslational modification by prolyl hydroxylation targets HIF-1α to the pVHL ubiquitination complex, leading to rapid proteasomal degradation (Ivan et al., 2001; Jaakkola et al., 2001).

In this report, we show the interaction of ARD1 with HIF-1α using the yeast two-hybrid system and characterize ARD1 as an important regulator of HIF-1α stability. We found that binding of ARD1 to HIF-1α was stronger under normoxic conditions than under hypoxic conditions. Moreover, forced expression of ARD1 diminished HIF-1α protein stability, whereas inhibition of ARD1 expression by transfection with an antisense ARD1 expression vector significantly increased protein stability. These results strongly suggest that ARD1 functions as a negative regulator of the HIF-1α protein.

Moreover, we characterized ARD1 as a protein acetyltransferase and HIF-1α protein as a substrate of ARD1.

ARD1 protein was discovered originally in *S. cerevisiae* as a component of the NAT complex and was found to have the ability to form a homodimer or heterodimer with NAT1 protein in *S. cerevisiae* (Park and Szostal, 1992). In mammalian cells, human ARD1 homolog was found in all tissues examined, although its functions and substrates have not been characterized (Tribioli et al., 1994).

Recently, TbDn-1 was isolated in mouse and found to be homologous to the yeast NAT1 protein, a subunit of the N-acetyltransferase complex that functions with the ARD1 protein in *S. cerevisiae* (Gendron et al., 2000). However, in mammalian cells, TbDn-1 alone shows acetyltransferase activity without ARD1 or other acetyltransferases (Gendron et al., 2000). Similarly, we found that the ARD1 protein itself acts as an acetyltransferase without a partner acetyltransferase and regulates HIF-1α stability. In fact, we found that neither TbDn-1 nor mouse NAT-1 homolog affected the stability of HIF-1α protein in human cells (data not shown). From these data, we suggest that ARD1 is a new mammalian acetyltransferase.

Several families of acetyltransferases are known, including PCAF/GCN5, p300/CBP, TAF250, SRC1, and MOZ (Kouzarides, 1999). These enzymes function as histone acetyltransferases and mainly exist in the nucleus; however, several nonhistone proteins, such as general transcription factors and DNA binding proteins, have been identified as substrates for PCAF and/or p300/CBP (Kouzarides, 2000). Recently, the acetylation of lysine residues in transcription factors by PCAF or p300/CBP has been described for p53 (Gu and Roeder, 1997), GATA-1 (Boyes et al., 1998; Hung et al., 1999), EKLF (Zhang and Bieker, 1998), MyoD (Sartorelli et al., 1999), TCF (Waltzer and Bienz, 1998), E2F1 (Martinez-Balbas et al., 2000), and Sp3 (Braun et al., 2001). The functional consequences of posttranslational modification by acetylation of these transcription factors appear to be quite varied (Kouzarides, 2000). For instance, the acetylation of p53, E2F1, and MyoD occurs close to the DNA binding domain and results in stimulation of DNA binding (Martinez-Balbas et al., 2000; Sartorelli et al., 1999; Huang et al., 2000). So far, functions for EKLF and TCF acetylation have not been established (Bannister and Miska, 2000).

Our results suggest that the HIF-1α protein is acetylated and that this modification is essential to the regulation of HIF-1α stability. The level of acetylation of HIF-1α was shown to gradually decrease as the length of hypoxic exposure time increases, which we found is partly due to the reduced expression of ARD1. Further, we showed ARD1 acetylates Lys532 in the ODD domain of HIF-1α by transferring acetyl group from Ac-CoA. The ODD domain encompasses sequences that mediate O_2-dependent ubiquitination of HIF-1α protein (Sutter et al., 2000) through the interaction with pVHL, which is the E3 ubiquitin-protein ligase (Cockman et al., 2000; Kamura et al., 2000) that targets HIF-1α for proteasomal degradation (Salceda and Caro, 1997). Binding of pVHL is dependent on hydroxylation of Pro402 and Pro564 in the ODD domain of HIF-1α through an enzymatic process that requires O_2 as well as iron and is inhibited by cobalt chloride (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001). Here, we report that the acetylation of Lys532 by ARD1 in the ODD domain is critical to the proteasomal degradation of HIF-1α. We found that a K532R mutant of HIF-1α was not acetylated by ARD1 protein, and that the mutant protein was stabilized and showed decreased interaction with pVHL during normoxia. Moreover, acetylation of the ODD domain by ARD1 increased the interaction with pVHL and stimulated ubiquitination of HIF-1α protein.

Previously, Tanimoto et al. (2000) examined three lysines (K532, K538, and K547) of N-TAD for degradation of HIF-1α during normoxia. These investigators showed that the K532R mutation stabilized HIF-1α during normoxia, and accordingly suggested that Lys532 is critical for the degradation of HIF-1α. Furthermore, HIF-1α, -2α, and -3α have two conserved prolines that are target sites for hydroxylation and pVHL binding in the ODD domain, whereas the lysine residue acetylated by ARD1 is conserved only in HIF-1α and -2α, not HIF-3α. In a previous report, HIF-1α and -2α were shown to be degraded under normoxic conditions, and protein levels increased in response to hypoxia, whereas the level of HIF-3α was not affected by O_2 concentration (Hara et al., 2001). Therefore, these data further support our finding that the acetylation of lysine residue plays a critical role in the degradation of HIF-α proteins in response to O_2 concentration.

Moreover, we found that HIF-1α-K532R was ubiquitinated at the same level as wild-type HIF-1α and that its binding to pVHL was not changed by ARD1. From these data, we suggest that Lys532 of HIF-1α is the target site for acetylation by ARD1, not a ubiquitination site. We also found that the acetylation of Lys532 in HIF-1α strongly increased the interaction with pVHL and induced pVHL-mediated ubiquitination.

Evaluating the collective evidence, we conclude that ARD1 acetylates Lys532 in the ODD domain of HIF-1α by direct interaction and plays a central role in HIF-1α stability by accelerating HIF-1α interaction with pVHL. Moreover, our data show that ARD1, as a negative regulator of HIF-1α stability, functions mainly under normoxic conditions due to the decreased ARD1 mRNA and a decreased affinity to HIF-1α under hypoxia. Therefore, the results reported here establish a key mechanism for the modulation of HIF-1α protein stability through acetylation.

Experimental Procedures

Materials

Sodium butyrate (NaBu) and trichostatin A (TSA) were purchased from Sigma and MG132 from Calbiochem. HIF-1α antibody was kindly provided by Dr. J.W. Park (College of Medicine, Seoul National University, Seoul, Korea). pVHL, Flag, and GFP antibodies were purchased from Pharmigen, Sigma, and CLONTECH, respectively. p53 and Ac-Lys antibodies were purchased from Upstate Biotechnology. Multiple Tissue Northern (MTN) blot was purchased from CLONTECH.

Plasmids and Recombinant Proteins

The full-length ARD1 expression vector was constructed by PCR and subcloned into pCMV-Tag (Stratagene). To construct antisense ARD1 vector, ARD1 cDNA fragment (1–308) was inserted into pCDNA3.1 (Invitrogen) in reverse orientation. To construct pET-ARD1 and GST-ARD1 for in vitro translation and bacterial induction, the ARD1 cDNA was inserted into pET-28 (Novagene) and pGEX-4T (Amersham Pharmacia Biotech). For luciferase assay, pBOS-

hHIF-1α, pBOS-hARNT, and pSV40pro-EpoHRE-Luc vectors were kindly provided by Dr. Fujii-Kuriyama (Tohoku University, Japan) (Ema et al., 1997), and mutated EpoHRE-Luc vector was provided by Dr. L. Eric Huang (NCI, National Institutes of Health, Bethesda, MD, USA). pGBT9-ODD, deletion mutants of HIF-1α, and GFP-HIF-1α vectors were prepared as previously described (Bae et al., 2002). For production of His fusion proteins, pET-ARD1 and pET-ODD vectors were transformed and His-ARD1 and His-ODD proteins were purified on a Talon metal affinity column (CLONTECH) according to the instructions of the manufacturer. All proteins were dialyzed using Sephadex G-25 column (Amersham Pharmacia Biotech).

Two-Hybrid Library Screening and Evaluation of Protein-Protein Interactions

Yeast strains for two-hybrid experiments were obtained from CLONTECH as components of the MATCHMAKER Two-hybrid System (CLONTECH). Yeast strains, SFY526 and HF7c, were used to assay protein-protein interactions and for library screening, respectively. Two-hybrid assays using the GAL4 system were performed according to the instructions of the manufacturer (CLONTECH).

GST Pull-Down Assay

The ^{35}S-labeled deletion mutants of HIF-1α were prepared by TNT system (Promega). GST-fusion proteins were purified as described (Shuman et al., 1997) and the GST pull-down assay was performed as described (Bae et al., 2002) using GST or GST-ARD1.

Cell Culture and Hypoxic Condition

HEK293 and HT1080 cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotics in a humidified 5% CO_2 incubator. For the generation of ARD1-overexpressing cells, HT1080 cells were transfected with pCMV-ARD1 and selected using G418 (Invitrogen). For the hypoxic condition, cells were incubated at a CO_2 level of 5% with 1% O_2 balanced with N_2 using a hypoxic chamber (Forma).

Coimmunoprecipitation

Transiently transfected HEK 293 cells or stably transfected HT1080 cells were treated for 6 hr or 2 hr under hypoxic conditions, respectively, before harvesting the cells. Preparation of protein extracts, immunoprecipitation, and Western blot were performed as described previously (Tanimoto et al., 2000).

Transient Transfection and Luciferase Assays

Five micrograms of plasmids was transfected to HEK293 cells, with proper recombinations of effector plasmids using calcium phosphate-mediated methods (Sambrook et al., 1989). The luciferase and β-galactosidase enzyme assays were performed as described (Bae et al., 2002).

Pulse-Chase Experiments

HT1080/HT1080-ARD1 cells and HEK 293 cells were labeled with 200 μCi of [^{35}S]methionine per plate in methionine-free DMEM (Invitrogen) under hypoxia for 4 hr. After labeling, cells were washed and chased with complete DMEM for the indicated amount of time in hypoxic chamber. Cells were subsequently subjected to immunoprecipitation as described above. Band intensities were measured using an autoradiopraph image analyzer (Fuji).

In Vivo Acetylation Assay

Sodium butyrate, an inhibitor of protein deacetylase, was added to growing HT1080 cells to a final concentration of 1 mM for 16 hr. Total cell extracts were isolated from sodium butyrate-treated cells and immunoprecipitated with 1 μg Ac-Lys antibody. The immunoprecipitates were subjected to SDS-PAGE and Western blot analysis with HIF-1α-specific antibody.

Protein Acetyltransferase Assay

Protein acetyltransferase assays were performed as described (Ito et al., 2001) using 100 ng purified bacterially expressed ODD protein and 40 ng ARD1 protein.

Site-Directed Mutagenesis

Site-directed mutagenesies of six lysine residues to arginine in both pET-ODD expression vector and Flag-tagged HIF-1α expression vector were performed using the Quick Change site-directed mutagenesis kit (Stratagene).

In-Gel Digestion and Mass Spectrometric Analysis

The acetylated ODD protein was excised from Coomassie-stained gel and purified as described (Song et al., 2000). Peptide mixtures were analyzed with MALDI-TOF MS using a delayed ion extraction and ion mirror reflector mass spectrometer (Voyager-DE STR; Applied Biosystems, Inc.). For interpretation of the mass spectra, we used the MS-Digest program available on the World Wide Web site of the University of California at San Francisco (http://prospector.ucsf.edu).

VHL-HIF-1α Binding Assay and In Vitro Ubiquitination Assay

For the binding assay, purified His-ODD protein (1 μg) was preincubated with HeLa cell extracts for 2 hr at 30°C and then binding assay was performed as described (Ohh et al., 2000) with [^{35}S]pVHL. For the ubiquitination assay, ^{35}S-labeled ODD (5 μl) was incubated in the presence of HeLa cell extracts (200 μg), and the ubiquitination assay was performed as described (Ohh et al., 2000). For the acetylation-combined experiments, an in vitro acetyltransferase assay was preperformed for 2 hr at 30°C.

Subcellular Fractionation of Cell Lysates

HT1080 and HT1080-ARD1 cells were incubated in 21% or 1% O_2 for 2 hr and separated into cytoplasmic and nuclear fractions as described (Kim et al., 1996).

Acknowledgments

We thank Dr. Fujii-Kuriyama for his gifts of the pBOS-hHIF-1α, pBOS-hARNT, and pSV40pro-EpoHRE-Luc vectors; Dr. Jong-Wan Park for the anti-HIF-1α antibody; Dr. L. Eric Huang for the mutated EpoHRE-Luc vector; and Dr. Byung Pal Yu for the critical reading of the manuscript. Financial support was provided by the National Research Laboratory Fund (2001-N-NL-01-C-015), the Ministry of Science and Technology, Korea (to K.-W.K.).

Received: June 3, 2002
Revised: September 17, 2002

References

Bae, M.K., Ahn, M.Y., Jeong, J.W., Bae, M.H., Lee, Y.M., Bae, S.K., Park, J.W., Kim, K.R., and Kim, K.W. (2002). Jab1 interacts directly with HIF-1α and regulates its stability. J. Biol. Chem. *277*, 9–12.

Bannister, A.J., and Miska, E.A. (2000). Regulation of gene expression by transcription factor acetylation. Cell. Mol. Life Sci. *57*, 1184–1192.

Barlev, N.A., Liu, L., Chehab, N.H., Mansfield, K., Harris, K.G., Halazonetis, T.D., and Berger, S.L. (2001). Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol. Cell *8*, 1243–1254.

Berra, E., Roux, D., Richard, D.E., and Pouyssegur, J. (2001). Hypoxia-inducible factor-1α (HIF-1α) escapes O_2-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm. EMBO Rep. *2*, 615–620.

Boyes, J., Byfield, P., Nakatani, Y., and Ogryzko, V. (1998). Regulation of activity of the transcription factor GATA-1 by acetylation. Nature *396*, 594–598.

Braun, H., Koop, R., Ertmer, A., Nacht, S., and Suske, G. (2001). Transcription factor Sp3 is regulated by acetylation. Nucleic Acids Res. *29*, 4994–5000.

Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science *294*, 1337–1340.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (2000). Hypoxia inducible factor-α binding and ubiquitination

by the von Hippel-Lindau tumor suppressor protein. J. Biol. Chem. *275*, 25733–25741.

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. Proc. Natl. Acad. Sci. USA *94*, 4273–4278.

Epstein, A.C.R., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., et al. (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43–54.

Gendron, R.L., Adams, L.C., and Paradis, H. (2000). Tubedown-1, a novel acetyltransferase associated with blood vessel development. Dev. Dyn. *218*, 300–315.

Gu, W., and Roeder, R.G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595–606.

Hara, S., Hamada, J., Kobayashi, C., Kondo, Y., and Imura, N. (2001). Expression and characterization of hypoxia-inducible factor (HIF)-3α in human kidney: suppression of HIF-mediated gene expression by HIF-3α. Biochem. Biophys. Res. Commun. *287*, 808–813.

Hewitson, K.S., McNeill, L.A., Riordan, M.V., Tian, Y.M., Bullock, A.N., Welford, R.W., Elkins, J.M., Oldham, N.J., Bhattacharya, S., Gleadle, J.M., et al. (2002). Hypoxia-inducible Factor (HIF) asparagine hydroxylase is identical to Factor Inhibiting HIF (FIH) and is related to the cupin structural family. J. Biol. Chem. *277*, 26351–26355.

Huang, L.E., Gu, J., Schau, M., and Bunn, H.F. (1998). Regulation of hypoxi-inducible factor 1 α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA *95*, 7987–7992.

Huang, S., Qiu, Y., Shi, Y., Xu, Z., and Brandt, S.J. (2000). P/CAF-mediated acetylation regulates the function of the basic helix-loop-helix transcription factor TAL1/SCL. EMBO J. *19*, 6792–6803.

Hung, H.L., Lau, J., Kim, A.Y., Weiss, M.J., and Blobel, G.A. (1999). CREB-binding protein acetylates hematopoietic transcription factor GATA-1 at functionally important sites. Mol. Cell. Biol. *19*, 3496–3505.

Ingram, A.K., Cross, G.A.M., and Horn, D. (2000). Genetic manipulation indicates that ARD1 is an essential N^{α}-acetyltransferase in *Tripanosoma brucei*. Mol. Biochem. Parasitol. *11*, 309–317.

Ito, A., Lai, C.H., Zhao, X., Saito, S., Hamilton, M.H., Appella, E., and Yao, T.P. (2001). p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. EMBO J. *20*, 1331–1340.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G. (2001). HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science *292*, 464–468.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., and Yu, A.Y. (1998). Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. Genes Dev. *12*, 149–162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., et al. (2001). Targeting of HIF-1 α to the von Hippel-Lindau ubiquitination complex by O_2-regulated prolyl hydroxylation. Science *292*, 468–472.

Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R.C., and Conaway, J.W. (2000). Activation of HIF-1α ubiqitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. Proc. Natl. Acad. Sci. USA *97*, 10430–10435.

Kim, C.G., Park, D., and Rhee, S.G. (1996). The role of carboxyl-terminal basic amino acids in Gq α-dependent activation, particulate association, and nuclear localization of phospholipase C-β1. J. Biol. Chem. *271*, 21187–21192.

Kouzarides, T. (1999). Histone acetylases and deacetylases in cell proliferation. Curr. Opin. Genet. Dev. *9*, 40–48.

Kouzarides, T. (2000). Acetylation: a regulatory modification to rival phosphorylation? EMBO J. *19*, 1176–1179.

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., and Whitelaw, M.L. (2002a). Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. Science *295*, 858–861.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruick, R.K. (2002b). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. *16*, 1466–1471.

Martinez-Balbas, M.A., Bauer, U.M., Nielsen, S.J., Brehm, A., and Kouzarides, T. (2000). Regulation of E2F1 activity by acetylation. EMBO J. *19*, 662–671.

Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-α chains activated by prolyl hydroxylation. EMBO J. *18*, 5197–5206.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271–275.

Ogryzko, V.V., Kotani, T., Zhang, X., Schiltz, R.L., Howard, T., Yang, X.J., Howard, B.H., Qin, J., and Nakatani, Y. (1998). Histone-like TAFs within the PCAF histone acetylase complex. Cell *94*, 35–44.

Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V., and Kaelin, W.G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the b-domain of the von-Hippel-Lindau protein. Nat. Cell Biol. *2*, 423–427.

Park, E.C., and Szostal, J.W. (1992). ARD1 and NAT1 proteins form a complex that has N-terminal acetyltransferase activity. EMBO J. *11*, 2087–2093.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehavy, L.E., Madan, A., Semenza, G.L., and Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. *14*, 34–44.

Richard, D.E., Berra, E., Gothie, E., Roux, D., and Poussegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1 α (HIF-1 α) and enhance the transcriptional activity of HIF-1. J. Biol. Chem. *274*, 32631–32637.

Salceda, S., and Caro, J. (1997). Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J. Biol. Chem. *272*, 22642–22647.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed., (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Sartorelli, V., Puri, P.L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J.Y., and Kedes, L. (1999). Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. Mol. Cell *4*, 725–734.

Semenza, G.L. (1999). Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu. Rev. Cell Dev. Biol. *15*, 551–578.

Semenza, G.L. (2000). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J. Appl. Physiol. *88*, 1474–1480.

Shuman, J.D., Cheong, J., and Coligan, J.E. (1997). ATF-2 and C/EBPalpha can form a heterodimeric DNA binding complex *in vitro*. Functional implications for transcriptional regulation. J. Biol. Chem. *272*, 12793–12800.

Song, E.J., Kim, Y.S., Chung, J.Y., Kim, E., Chae, S.K., and Lee, K.J. (2000). Oxidative modification of nucleoside diphosphate kinase and its identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Biochemistry *39*, 10090–10097.

Sutter, C.H., Laughner, F., and Semenza, G.L. (2000). HIF-1α protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutation. Proc. Natl. Acad. Sci. USA *97*, 4748–4753.

Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. (2000). Mechanism of regulation of the hypoxia-inducible factor-1 α by the von Hippel-Lindau tumor suppressor protein. EMBO J. *19*, 4298–4309.

Tribioli, C., Mancini, M., Plassart, E., Bione, S., Rivella, S., Sala, C., Torri, G., and Toniolo, D. (1994). Isolation of new genes in distal Xq28: transcriptional map and identification of a human homologue of the ARD1 N-acetyltransferase of *Saccharomyces cerevisiae*. Hum. Mol. Genetics *3*, 1061–1067.

Waltzer, L., and Bienz, M. (1998). *Drosophila* CBP repressed the transcription factor TCF to antagonize Wingless signaling. Nature *395*, 521–525.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. USA *92*, 5510–5514.

Zhang, W., and Bieker, J.J. (1998). Acetylation and modulation of erythroid Kruppel-like factor (EKLF) activity by interaction with histone acetyltransferases. Proc. Natl. Acad. Sci. USA *95*, 9855–9860.