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Sumoylation increases HIF-1α stability and its transcriptional activity

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Abstract

HIF-1 is closely involved in various biological processes, including angiogenesis, energy metabolism, and cell survival. HIF-1 consists of an oxygen-sensitive HIF-1α and oxygen-insensitive HIF-1β. Oxygen-sensitive HIF-1α is subjected to post-translational modifications such as hydroxylation, ubiquitination, and acetylation, which are related to the regulation of its stability. In this present study, we found that the ectopic expression of SUMO-1 increased HIF-1α stability by the co-transfection study with HIF-1α and SUMO-1. Furthermore, the ectopic expression of SUMO-1 enhanced the transcriptional activity of HIF-1α. In the subsequent immunoprecipitation assay, SUMO-1 was co-immunoprecipitated with HIF-1α, implying that HIF-1α is covalently modified by SUMO-1. Thereafter, using a series of lysine mutants in the ODD domain, we found that HIF-1α was sumoylated at Lys^{391} and Lys^{477}, suggesting that sumoylation at these two lysine residues enhances HIF-1α stability by possibly modulating other post-translational modifications. Altogether, we demonstrate that HIF-1α is upregulated through SUMO-1 modification at Lys^{391}/Lys^{477} residues, which may stabilize HIF-1α and enhance its transcriptional activity.

Keywords: SUMO; HIF; Hypoxia; Ubiquitination; Acetylation

HIF-1 is a key regulator of many biological processes, including angiogenesis, energy metabolism, and cell survival [1,2]. HIF-1 is composed of HIF-1α and HIF-1β subunits [3]. HIF-1α expression is regulated depending on oxygen concentration, whereas HIF-1β is constantly expressed. As cells are exposed to relatively lower oxygen, HIF-1α is dramatically increased. Over a decade of years, the regulatory mechanisms of HIF-1α have been extensively studied. Recent progresses have revealed that HIF-1α stability and its activity are tightly regulated through a cascade of post-translational modifications in response to oxygen availability. Post-translational modifications such as ubiquitination, sumoylation, and acetylation have fervently been addressed, in particular, on lysine residues as significant modifiers for HIF-1α activity. These modifiers might undergo a more complex interplay through competitive interaction at lysine residues of HIF-1α.

HIF-1α is rapidly degraded via pVHL-mediated ubiquitin–proteasome pathway under normoxia [4–6]. In oxygenated cells, HIF-1α is hydroxylated in the proline residue in the oxygen-dependent degradation (ODD) domain, the association of pVHL E3 ubiquitin ligase and HIF-1α is facilitated, and then targeted to the ubiquitin–proteasome complex for rapid proteolysis of HIF-1α [7–9]. The hydroxylation is catalyzed at two

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prolyl residues (Pro^{402}, Pro^{564} of human HIF-1α) by prolyl-4-hydroxylase domain (PHD) protein 1-3 [10,11]. Moreover, hydroxylation of Asn^{803} in the C-TAD of HIF-1α is exerted by HIF asparaginyl hydroxylase (FIH). FIH hydroxylation of HIF-1α interferes with the recruitment of a coactivator, p300. Therefore, HIF hydroxylases allow HIF-1α to sense and respond to oxygen tension [12–14].

The arrest-defective-1 (ARD1), an *N*-acetyltransferase, seems to be another important mediator of HIF-1α regulation. Lysine residue works as a substrate for ARD1 modification. The acetylation of Lys^{532} of the ODD domain by ARD1 favors recruitment of the von Hippel–Lindau protein (pVHL) and likely enhances the proteasomal degradation of HIF-1α, whereas the blockade of acetylation by mutation of Lys^{532} decreases interaction with pVHL and stabilized HIF-1α [15].

Recently, sumoylation has also come to the stage of modification of transcriptional and non-transcriptional factors [16,17]. Several ubiquitin-like protein families that share similarity with ubiquitin have been identified. Of which, the small ubiquitin-related modifier-1 (SUMO-1) is around 12 kDa protein that covalently linked to lysine residue of consensus motif ψKxE (ψ for hydrophobic amino acid and x for any amino acid) [18]. Modification by SUMO-1, sumoylation, is thought to be enzymatically similar to ubiquitination in terms of requiring an E1 heterodimer, an E2 conjugator, and an E3 ligase. In contrast to ubiquitination that undergoes proteasomal degradation, sumoylation has diverse effects. For instance, sumoylation of RanGAP1, PML, and Sp100 alters their subcellular localizations [19–22]. Sumoylation regulates transcriptional activity positively or negatively. The transcriptional activity of p53 increased after SUMO modification. In contrast to p53, the activity of androgen receptor by sumoylation was decreased [23,24]. Since SUMO and ubiquitin bind lysine residues, there is a possibility that sumoylation competes with ubiquitination on the same lysine residue. In fact, there are several reports that sumoylation of IκB and Smad4 increases their stabilities by competing with ubiquitination followed by proteasomal degradation [25].

As another form of HIF-1α modification, the most recent report has been shown that SUMO-1 expression is upregulated in response to hypoxia in adult mouse brain and heart [26]. In addition, Shao et al. [26] have suggested that SUMO-1 co-localizes and co-immunoprecipitates with HIF-1α in the nucleus. In advance, here, we found that HIF-1α stability and its transcriptional activity are upregulated by SUMO modification at Lys^{391} and Lys^{477} residues of HIF-1α.

Materials and methods

Plasmid. For the luciferase assay, pBOS-HIF-1α and pSV40-EpoHRE-Luc were kindly provided by Dr. Fujii-Kuriyama (Tohoku University, Japan). For the in vitro sumoylation assay, SUMO-1 and Ubc9 plasmids, and SAE 1/2 proteins are gently provided by Dr. Chin-Ha Chung (Seoul National University, Korea).

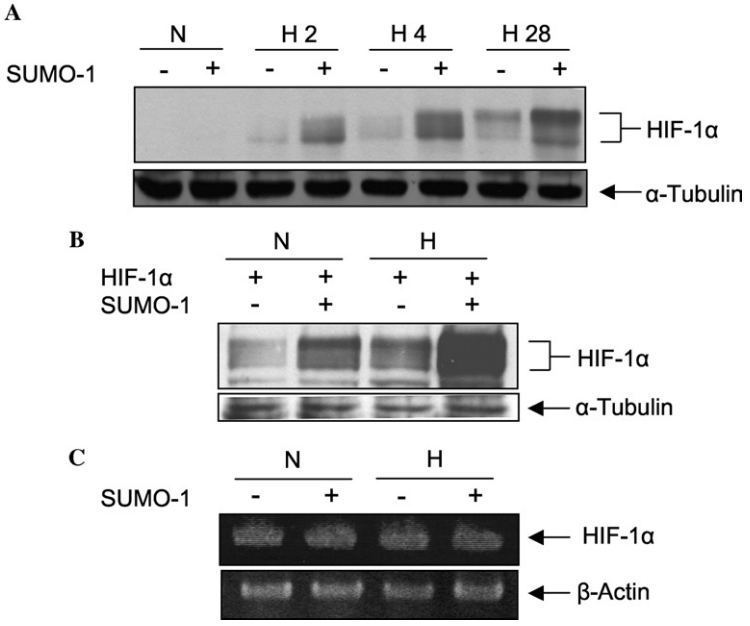

Fig. 1. SUMO-1 stabilizes HIF-1α. (A) HeLa cells were transfected with SUMO-1 and exposed to 1% O_2 (H) for 2, 4, and 28 h or to 21% O_2 (N). The endogenous HIF-1α protein level was examined by Western blot analysis. (B) HEK 293T cells were co-transfected with pBOS-hHIF-1α and SUMO-1. Cells were exposed to 21% (N) or 1% O_2 (H) for 16 h and subjected to Western blot analysis of HIF-1α. (C) HeLa cells were transfected with SUMO-1 and exposed to 1% O_2 (H) for 4 h or to 21% O_2 (N). HIF-1α mRNA level was examined by RT-PCR.

Cell culture and hypoxic condition. 293T cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD, USA) and 1% antibiotics. For hypoxic condition, cells were incubated at 5% CO_2 level with 1% O_2 balanced with N_2 in hypoxic chamber (Forma, Marietta, OH, USA).

Antibodies. Mouse monoclonal antibody against HIF-1α was purchased from BD Bioscience (San Diego, CA, USA). Rabbit polyclonal antibodies against VEGF and SUMO-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Transient transfection and luciferase assay. Five micrograms of plasmids was transfected to 293T cells with proper recombinations of effector plasmids, pSV40promoter-EpoHRE-Luc reporter, pCMV-β-gal, pBOS-hHIF-1α, and pCMV-SUMO1 using calcium phosphate-mediated methods. After transfection, cell lysates were analyzed for luciferase activity using an assay kit (Promega) and a luminometer (Turner Design). Extracts were also applied to the β-galactosidase enzyme assay. Each extract was assayed three times, and the mean relative light unit was normalized by values obtained from an extract prepared from empty vector-transfected cells. The relative luciferase activity was calculated as relative light units/β-galactosidase.

Western blotting. Cell extracts were prepared and aliquots (30 μg) were loaded into 6–12% SDS–PAGE gel and transferred to protein nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was incubated in blocking buffer (5% skim milk in PBS-T) at room temperature (RT). Then the filter was incubated with specific primary antibody at 4 °C for overnight and washed with PBS-T (0.1% Tween 20 in PBS) three times every 10 min, followed by incubation with secondary antibody at RT for 1 h. Immunoreactive bands were visualized using chemiluminescent reagent as recommended by Amersham Pharmacia Biotechnology.

In vivo sumoylation assay. HeLa cells were treated for 2 h under hypoxic condition before harvesting the cells. The cell pellet was resuspended in 200 μl of whole cell extract buffer (10 mM Hepes, pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 5% glycerol, and 1 mM DTT) supplemented with 10 mM *N*-ethylmaleimide and 10 mM iodoacetamide, followed by centrifugation for 30 min at 15,000 rpm. Then, 1 mg of total cell proteins was incubated with SUMO-1 antibody, protein A–Sepharose in TEG buffer for 12 h at 4 °C under rotation. After rapid centrifugation, the resulting Sepharose pellets were washed with supplemented TEG buffer, and the bound proteins were solubilized by addition of SDS sample buffer.

In vitro transcription/translation. The TNT T7 Quick-coupled Transcription/Translation System (Promega) was used for in vitro transcription and translation. One microgram of plasmid DNA, 40 μl of the TNT Quick Master Mix, and 20 μCi [^{35}S]methionine were combined and incubated at 30 °C for 90 min.

Site-directed mutagenesis. Site-directed mutagenesis of nine lysine residues to arginine was performed using the Quick Change site-directed mutagenesis kit (Stratagene).

In vitro sumoylation assay. ^{35}S-Labeled in vitro translated HIF-1α protein (5 μl) was incubated with 10 μg of purified SUMO-1, 5 μg Ubc9, and 1.5 μg SAE1/2 including ATP regenerating buffer (10 mM creatine phosphate, 3.5 U/ml creatine phosphokinase, 0.6 U/ml inorganic phosphatase) at 37 °C for 2 h.

Results

HIF-1α stability is increased by SUMO-1

Since sumoylation has been known to regulate activities of transcription factors positively and negatively, we examined whether SUMO-1 could regulate HIF-1α stability. We observed that SUMO-1 significantly increased the expression of HIF-1α, after SUMO-1 was transfected into HeLa cells (Fig. 1A). The expression of endogeneous HIF-1α was upregulated in a time-dependent manner. Co-transfection of HIF-1α and SUMO-1 confirmed HIF-1α upregulation by SUMO-1 under both normoxic and hypoxic conditions in 293T cells (Fig. 1B). In subsequent RT-PCR experiments (Fig. 1C), the expression level of HIF-1α mRNA was unchanged, implying that SUMO-1 is likely to stabilize the HIF-1α protein.

Transcriptional activity of HIF-1α is upregulated by SUMO-1

We next examined the possibility of SUMO-1 in upregulating the transcriptional activity of HIF-1α using an HRE luciferase reporter gene, pSV40promoter-EpoHRE-Luc. Enforced expression of SUMO-1

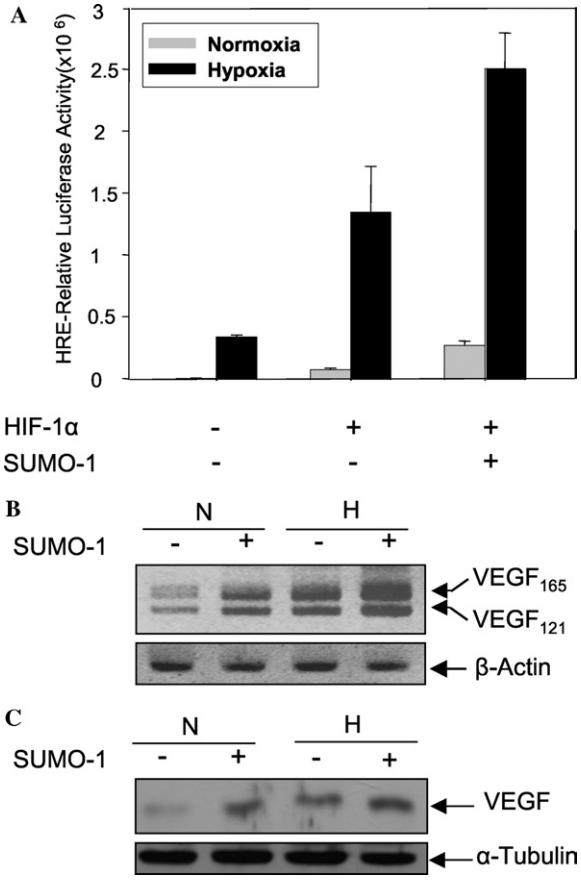

Fig. 2. SUMO-1 increases the transcriptional activity of HIF-1α. (A) HEK 293T cells were co-transfected with pSV40pro-EpoHRE-Luc (1 μg), pBOS- hHIF-1α (1 μg), and pCMV-SUMO-1 (2 μg) or pCMV alone as indicated. Transfected cells were incubated under 21% O_2 or 1% O_2 for 16 h. The mean and standard deviation based on three independent transfections are shown. (B,C) After transfection of HeLa cells with SUMO-1, RT-PCR and Western blot analysis were performed using specific primers for VEGF and anti-VEGF antibody, respectively.

and HIF-1α led to markedly increase EpoHRE promoter activity under normoxia and hypoxia, compared to that of HIF-1α alone (Fig. 2A). In addition, the effect of HIF-1α upregulation on its transcriptional activity was confirmed by RT-PCR and immunoblotting, as shown in Figs. 2B and C. The ectopic expression of SUMO-1 increased the mRNA and protein levels of VEGF under both normoxia and hypoxia.

HIF-1α is modified by SUMO-1 in vivo and in vitro

The above results are consistent with the possibility that HIF-1α stabilization by SUMO-1 is due to the interaction between SUMO-1 and HIF-1α. When the endogenous SUMO-1 protein or HIF-1α was immunoprecipitated, HIF-1α was detected by Western blot analysis (Fig. 3A). This result suggests that HIF-1α is covalently modified by SUMO-1 in vivo.

Analysis of amino acid sequences nearby and in the ODD domain (378–603 amino acids, referred to as ODD) showed three lysine residues of the putative sumoylation sequence, ψKxE; Lys^{391}, Lys^{477}, and Lys^{532} (Fig. 3B). Subsequently, we constructed several plasmids containing this sequence of HIF-1α and translated with rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Sumoylation assay was performed by the addition of SUMO-1, SAE 1/2, and Ubc9 to the translated ODD protein. As shown in Fig. 3C, two bands of the modified ODD were detected: the first one at 40 kDa; the second one at 52 kDa. This result suggests that HIF-1α has, at least, two sumoylation sites.

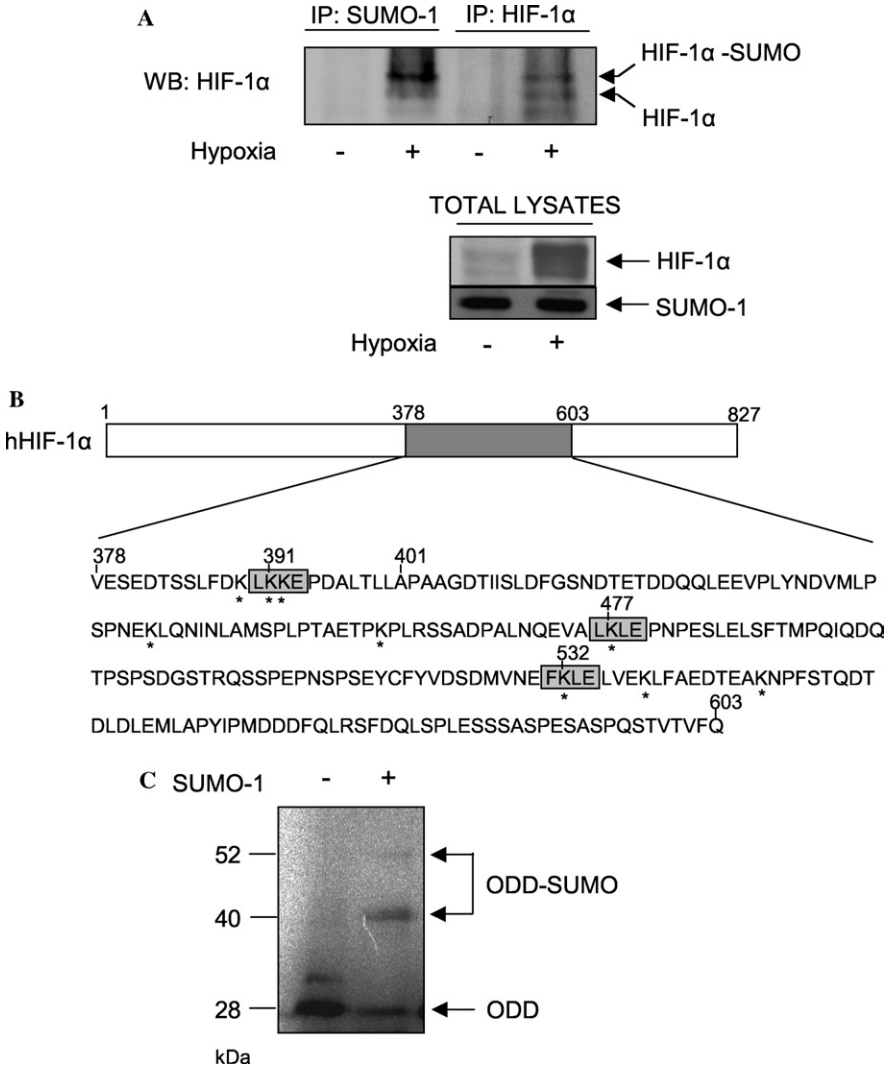

Fig. 3. HIF-1α is modified by SUMO-1 in vivo and in vitro. (A) Endogenous SUMO-1 and HIF-1α in HeLa cells were immunoprecipitated with anti-SUMO1 or anti-HIF-1α antibody, and co-immunoprecipitated HIF-1α was detected by Western blot analysis (upper). In total lysates, endogenous HIF-1α and SUMO-1 were identified by Western blot analysis (lower). (B) Sequence nearby and in the ODD domain (378–603 amino acids, referred to as ODD) of HIF-1α. The asterisk denotes lysine (K) residues that are substituted for arginine (R) residues. The box denotes consensus sequence ψKxE for SUMO modification. (C) pET-ODD was translated in the presence of [^{35}S]methionine and analyzed by autoradiography after in vitro sumoylation assay.

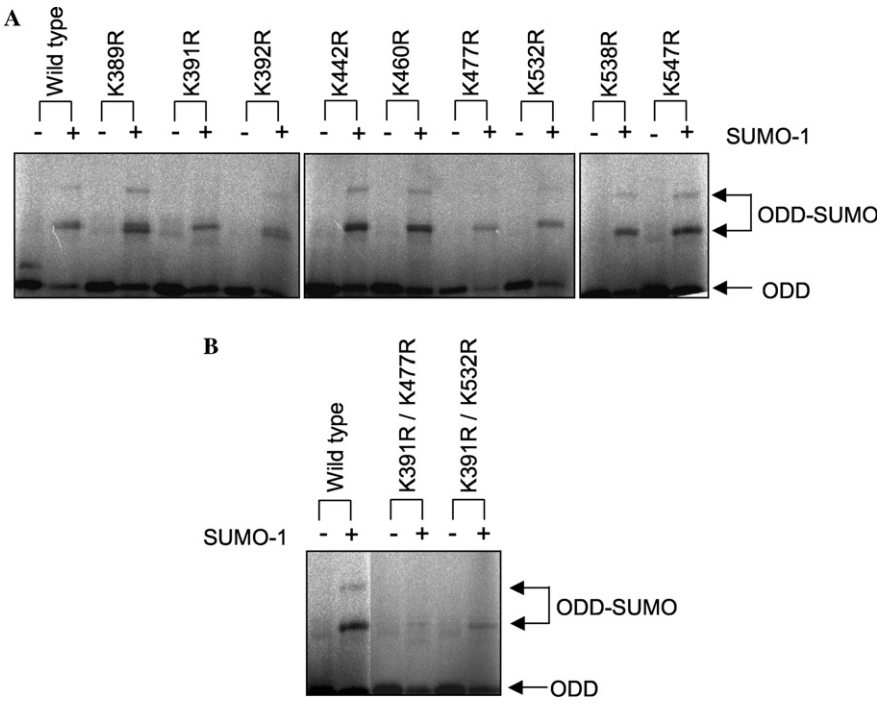

Fig. 4. Lys[391] and Lys[477] are SUMO modification sites of HIF-1α. (A) In vitro sumoylation assay was performed for a wild type or mutants of the ODD domain (substitution of lysine to arginine). (B) K391R/K477R and K391R/K532R double mutants were also applied to the sumoylation assay.

Lysine residues at the position 391 and 477 of HIF-1α are required for SUMO-1 modification

To identify substrate residues for sumoylation, we generated nine lysine (K) mutants in the ODD domain, including K391, K477, and K532, which were substituted for arginine residues (R) as depicted in Fig. 3B. Interestingly, in vitro sumoylation assay showed that a sumoylated 52 kDa ODD band disappeared and the intensity of a sumoylated 40 kDa ODD band was subtly decreased in K391R and K477R mutants. However, other lysine mutants showed no effect (Fig. 4A), except that K532R mutant seemed to show a little reduction in the intensity of a 52 kDa ODD band. Therefore, to confirm the sumoylated sites, double mutants K391R/K477R and K391R/K532R were generated and assayed for sumoylation. As shown in Fig. 4B, K391R/K477R double mutant led to abolish the two sumoylated HIF-1α bands. However, K391R/K532R double mutant still maintained the 40 kDa ODD band, suggesting that Lys[532] is not modified by SUMO-1. These results indicate that Lys[391] and Lys[477] of HIF-1α are targets of SUMO-1 modification.

Discussion

HIF-1α is a master regulator among a lot of transcription factors and functions depending on oxygen tension. HIF-1α is rapidly degraded via VHL-ubiquitin proteasomal pathway under normoxia, whereas HIF-1α is stabilized and exerts its tremendous transcriptional activities under hypoxia. Therefore, HIF-1α stabilization is critical for the events mediated by hypoxia.

Here we have demonstrated that the protein level and transcriptional activity of HIF-1α are upregulated by SUMO-1. The protein level of HIF-1α was dramatically increased in the overexpressed SUMO-1 under normoxia and hypoxia. Moreover, not only the transcriptional activity of HIF-1α but also VEGF expression was markedly increased in the presence of SUMO-1. These data led us to contemplate a possibility of the increase of HIF-1α stability by SUMO-1 modification. The possibility was proved by the result that HIF-1α was covalently modified by SUMO-1 in vivo and in vitro (Fig. 3). Supporting our findings, Shao et al. [26] have also demonstrated in the recent report that hypoxia-induced SUMO-1 interacts with HIF-1α and results in HIF-1α sumoylation. Therefore, the sumoylated HIF-1α is likely to be stabilized and located in the nucleus and to contribute in enhancing the transcriptional activity of HIF-1α.

Significance of ODD domain for the stability of HIF-1α has been focused, followed by a rapid advance to gain insight into HIF-1α regulation in response to oxygen tension. Lysine residues in the ODD domain are likely to serve in the process of ubiquitination, acetylation, and sumoylation. Until now, however, it remains unclear which lysine residues are in charge of the process of ubiquitination. Contrast to ubiquitination, it has almost recently been reported that Lys[532] residue is acetylated by ARD1, *N*-acetyltransferase, which increases the

proteosomal degradation of HIF-1α. It is tempting to speculate that modification of lysine residues in the ODD domain might function coordinately and competitively to turn HIF-1α on and off.

To identify which lysines are in charge of HIF-1α sumoylation, we constructed a series of mutants on the lysine residues in the ODD domain (378–603 amino acids, referred to as ODD) and applied to the in vitro sumoylation assay (Fig. 4A). From conventionally known consensus sequences ψKxE (ψ for hydrophobic amino acid and x for any amino acid) for SUMO modification, it was considered that Lys^{391}, Lys^{477}, and Lys^{532} of HIF-1α might be modified by SUMO-1. However, we found that only Lys^{391} and Lys^{477} of HIF-1α were the sites for SUMO-1 modification (Fig. 4). These results were confirmed by double mutant K391R/K477R.

It has been suggested that sumoylation of lysine residues may prevent proteins from ubiquitination and subsequent proteosomal degradation in a specific way. With these modifications, acetylation in lysine residues can alter protein functions. In our previous report, we have demonstrated that Lys^{532} of HIF-1α is one of the ψKxE consensus motifs and at which ARD1 acetylates and destabilizes HIF-1α. Hence, we assumed that the Lys^{532} residue might also be one of the targets for sumoylation. However, Lys^{532} is unlikely to be a SUMO modification site because a mutant K532R and a double mutant K391R/K532R did not abolish the slowly migrating band (Fig. 4). These results suggest that Lys^{391} and Lys^{477} residues may be involved in the competition between ubiquitin and SUMO-1 for HIF-1α stability, not Lys^{532} residue that is acetylated by ARD1. Therefore, it might be attractive to elucidate the role of acetylated HIF-1α by ARD1 in regulating its sumoylation and ubiquitination.

Taken together, HIF-1α is sumoylated by SUMO-1, which leads to an increase in HIF-1α stability and its transcriptional activity, implying that SUMO-1 may counteract ubiquitin. Moreover, we suggest that sumoylation occurs at Lys^{391}/Lys^{477} residues, but not Lys^{532} residue. Therefore, two lysine residues, Lys^{391} and Lys^{477}, provide unique post-translational modification sites for HIF-1α stability through sumoylation.

Acknowledgments

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