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# Histone Deacetylase Inhibitors Induce VHL and Ubiquitin-Independent Proteasomal Degradation of Hypoxia-Inducible Factor 1α

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**Adaptation to hypoxic microenvironment is critical for tumor survival and metastatic spread. Hypoxia-inducible factor 1α (HIF-1α) plays a key role in this adaptation by stimulating the production of proangiogenic factors and inducing enzymes necessary for anaerobic metabolism. Histone deacetylase inhibitors (HDACIs) produce a marked inhibition of HIF-1α expression and are currently in clinical trials partly based on their potent antiangiogenic effects. Although it has been postulated that HDACIs affect HIF-1α expression by enhancing its interactions with VHL (von Hippel Lindau), thus promoting its ubiquitination and degradation, the actual mechanisms by which HDACIs decrease HIF-1α levels are not clear. Here, we present data indicating that HDACIs induce the proteasomal degradation of HIF-1α by a mechanism that is independent of VHL and p53 and does not require the ubiquitin system. This degradation pathway involves the enhanced interaction of HIF-1α with HSP70 and is secondary to a disruption of the HSP70/HSP90 axis function that appears mediated by the activity of HDAC-6.**

The transcriptional complex hypoxia-inducible factor 1 (HIF-1) plays a key role in the cellular adaptations to the lack or deficiency of oxygen supply (17). These adaptations are critical in solid tumors, where the process of tumor expansion exceeds the development of blood vessels, resulting in a hypoxic tumor microenvironment (10, 18, 52). Measurements of oxygen using either direct or indirect techniques have shown that tumor cells may exist under extremely hypoxic conditions, where $pO_2$ values below 2 to 3 torr are not uncommon (12). Successful tumor progression depends on physiologic adaptations and selection of genetic alterations that will ensure tumor cell survival in this unfavorable hypoxic environment. These tumor adaptations include the activation of HIF-1-dependent genes that control angiogenesis and facilitate anaerobic glycolysis (47). Thus, drugs that inhibit HIF-1 expression have great potential as antitumor agents. The activation of HIF-1 is mediated primarily by the hypoxic stabilization of its HIF-1α subunit. HIF-1α is constitutively transcribed and translated, but in normoxic conditions the HIF-1α protein is rapidly degraded by the ubiquitin-proteasome system (19, 25, 26, 45). Thus, no HIF-1α proteins are found in well-oxygenated cells whereas its levels increase promptly following the onset of hypoxia. The degradation of HIF-1α is mediated by its interactions with the von Hippel-Lindau (VHL) protein, a tumor suppressor that acts as a ubiquitin ligase (E3) (9, 37). This interaction explains the high level of HIF-1α expression and the high degree of vascularization observed in VHL-deficient tumors (30). In turn, the interaction between HIF-1α and VHL depends on the enzymatic hydroxylation of two prolyl residues (P402 and P564) in the oxygen degradation domain (ODD) of HIF-1α (21, 22, 36). These hydroxylation reactions are oxygen dependent and are mediated by prolyl hydroxylase enzymes whose activities also depend on the availability of $Fe^{++}$, vitamin C, and oxoglutarate (reviewed in reference 40).

Despite the central importance of hydroxylases in sensing oxygen and regulating HIF-1α, an array of factors, including cytokines, growth factors, and oncogenic mutations, have also been shown to control HIF-1α expression, even under normoxic conditions (14, 49). The stimulation of HIF-1α by these additional pathways explains the finding of HIF-1α protein in nonhypoxic areas of tumors and may contribute to the development of early tumor angiogenesis, before the onset of hypoxia (48, 54). While some of the mechanisms involved in the non-oxygen-dependent control of HIF-1α are known, such as the case of VHL mutations, less is known regarding the contribution of growth factors and cytokines. Proposed mechanisms involve the increased translation of HIF-1α mRNA or the induction of posttranslational modifications such as phosphorylation or acetylation that may affect HIF-1α protein stability (reviewed in reference 6).

Although their mechanism of action is unclear, histone deacetylase inhibitors (HDACIs) have been reported to reduce HIF-1α expression and are currently in clinical trials partly due to their potent antiangiogenic effects (29, 41). These compounds are well known to affect gene expression through the hyperacetylation of histones. However, their effects are not restricted to these proteins: $N^{\varepsilon}$ acetylation of transcription factors is emerging as an important mechanism of regulating transcription factor activity by affecting their DNA-binding affinity, transactivation activity, stability, or subcellular localization (35, 53). Two opposing group of enzymes, acetylases and deacetylases, modulate the acetylation status of histone and nonhistone proteins (53).

The role of acetylation in controlling HIF-1α function is a matter of controversy. An earlier report by Kim et al. (29) suggested that HDAC inhibitors downregulated HIF-1α by

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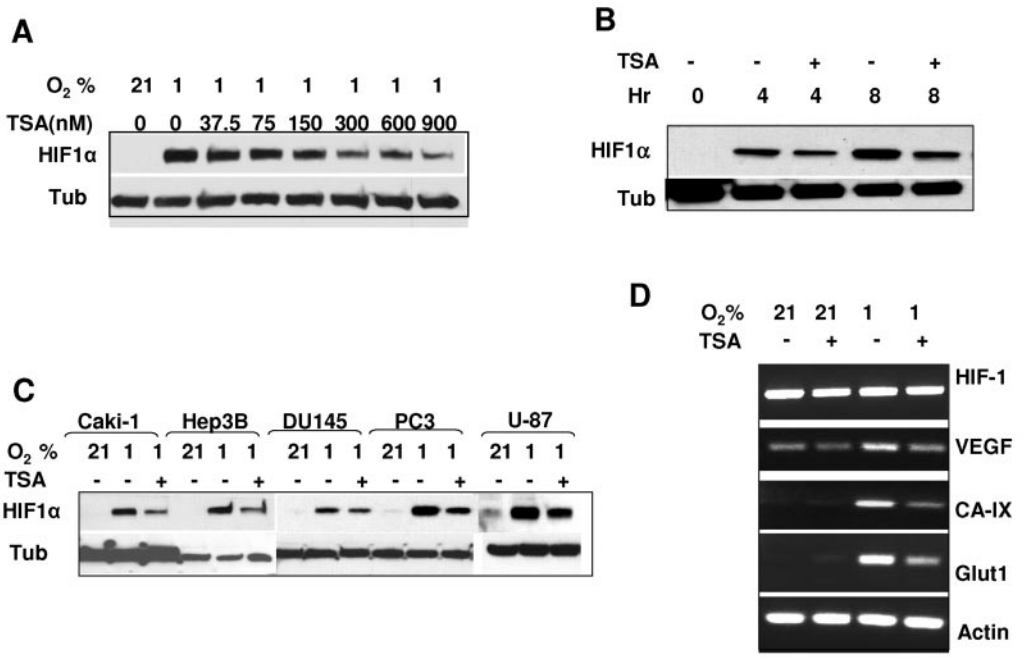


FIG. 1. TSA represses HIF-1α induction in response to hypoxia. A. Dose responses. U-87 cells were exposed to hypoxia (1% $O_2$) for 8 h in the presence of increasing concentrations of TSA. Cell lysates (40 μg) were analyzed by Western blotting using anti-HIF-1α and tubulin (Tub) monoclonal antibodies. B. Time course. U-87 cells were exposed to 300 nM of TSA and 1% $O_2$ for 4 or 8 h, and HIF-1α levels were analyzed by Western blotting. C. Tumor cell lines. Different cell lines were exposed to dimethyl sulfoxide or 300 nM of TSA for 8 h under 1% $O_2$. D. Effect of TSA on gene expression. Total RNA from U-87 cells exposed to dimethyl sulfoxide or TSA for 8 h under conditions of 21% or 1% $O_2$ was analyzed by RT-PCR using primers described in Materials and Methods. VEGF, vascular endothelial growth factor.

upregulating p53 and VHL, thus promoting HIF-1α degradation. Later, the same group postulated the acetylation of K532 of HIF-1α by the acetyl-transferase ARD1 as a novel mechanism that controls HIF-1α stability (23). It was proposed that acetylation of HIF-1α would increase its affinity for VHL and thereby enhance HIF-1α ubiquitination and proteasomal degradation. However, ARD1 was originally described in *Saccharomyces cerevisiae* as an $N^{\alpha}$-acetyltransferase and its ability to $N^{\varepsilon}$-acetylate HIF-1α has not been confirmed (2, 5, 13, 43). Herein, we present evidence that HDACIs decrease HIF-1α and the expression of hypoxia-regulated genes by a pathway that does not involve its association with VHL or p53, as suggested by Kim et al. and Jeong et al. (23, 29), but rather by affecting its interactions with the HSP70/HSP90 chaperone axis and inducing its ubiquitin-independent proteasomal degradation.

## MATERIALS AND METHODS

**Cell cultures.** Except when indicated, cell lines were obtained from the ATCC and cultured according to their instructions. Other cell lines were generously provided by the following investigators: RCC4 $VHL^{-/-}$ and $VHL^{+/+}$ cells were provided by P. Ratcliffe (Oxford University, Oxford, England); C2 cells were provided by L. Neckers (National Cancer Institute); HCT116 cells ($p53^{+/+}$ and $p53^{-/-}$) were provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD); and A549 cells overexpressing HDAC-6 and 293T HDAC-6 knockdown cells were provided by T. P. Yao (Duke University, Durham, NC). The Ts20 cells containing a temperature-sensitive E1 were supplied by H. L. Ozer (Rutgers University) and were utilized as described before (45). Hypoxia (1% $O_2$, 5% $CO_2$) conditions were created in an oxygen station (In $VIVO_2$; Ruskin Tech).

**Chemicals and reagents.** Common chemicals, solvents, and general reagents were obtained from Sigma. HDACI trichostatin A (TSA) was from Bio-Mol (Plymouth Meeting, PA), and SAHA (suberoylanilide hydroxamic acid) was from Bio-Vision (Mountain View, CA). The proteasome inhibitor MG132 (Z-LLL-CHO) was from Sigma. Epoxomicin and caspase and calpain inhibitors Z-VAD-FMK and Z-Leu-Leu-CHO were from BioMol.

**Immunoblots and immunoprecipitations.** Antibodies were obtained as follows: monoclonal anti-hHIF-1α was from BD Biosciences (San Diego, CA); anti-mHIF-1α was from R&D Systems (Minneapolis, MN); HDAC-6, p53, alpha-tubulin, and actin were from Santa Cruz (Santa Cruz, CA); monoclonal anti-HSP70 and -HSP90 were from Cell Signaling (Canada); and anti-acetyllysine antibodies were from Cell Signaling and Upstate Biotechnology, Inc. (Lake Placid, NY). Cell lysates for Western blot analyses were obtained by homogenizing cells in 8 M urea buffer (8 M urea in 10 mM Tris [pH 6.8], 1% sodium dodecyl sulfate, 5 mM dithiothreitol) in the presence of a 1× protein inhibitor mix with a portable Ultra-Turrax homogenator. Western blotting was performed as previously described using horseradish peroxidase-conjugated secondary antibodies and an ECL Plus kit from Amersham (46). Immunoprecipitations were performed essentially as described previously (46), utilizing lysis buffer containing 1% Triton 100, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM $Na_3VO_4$, and 1 mM phenylmethylsulfonyl fluoride in the presence of a 1× protease-inhibitor mix. Primary antibody incubation was performed for 4 h at 4°C followed by precipitation with protein A-Sepharose (Pierce). Precipitates were extensively washed in lysis buffer and utilized for immunoblotting.

**Plasmids and transfections.** Full-length HIF-1α, HIF-ODD (amino acids [aa] 393 to 580) and HIF-ODD-K532R, HIF-Δ392–575 (originally provided by G. Semenza), and HIF-1α P402A/P564G (originally provided by P. Ratcliffe) were cloned in the pShooter vector containing a nuclear localization signal and a Myc tag (Invitrogen). Transfections were carried out as previously described (46) using Lipofectamine 2000 (Invitrogen).

**RNA extraction and RT-PCR.** Total RNA was extracted from cultured cells using a kit (RNeasy) from QIAGEN (Valencia, CA). Following digestion with RNase-free DNase, reverse transcription (RT) was performed using an RT kit from La Roche. Semiquantitative PCR was optimized for each individual target. Primers utilized were as follows: for β-actin (409 bp), the forward primer was ACCAACTGGGACGACATGGAGAAA and the reverse primer was TTA ATGTCACGCACGATTTCCCGC; for VEGF165 (310 bp), the forward primer was TTTCTGCTGTCTTGGGTGCATTGG and the reverse primer was TCT GCATGGTGATGTTGGACTCCT; for HIF-1α (564 bp), the forward primer

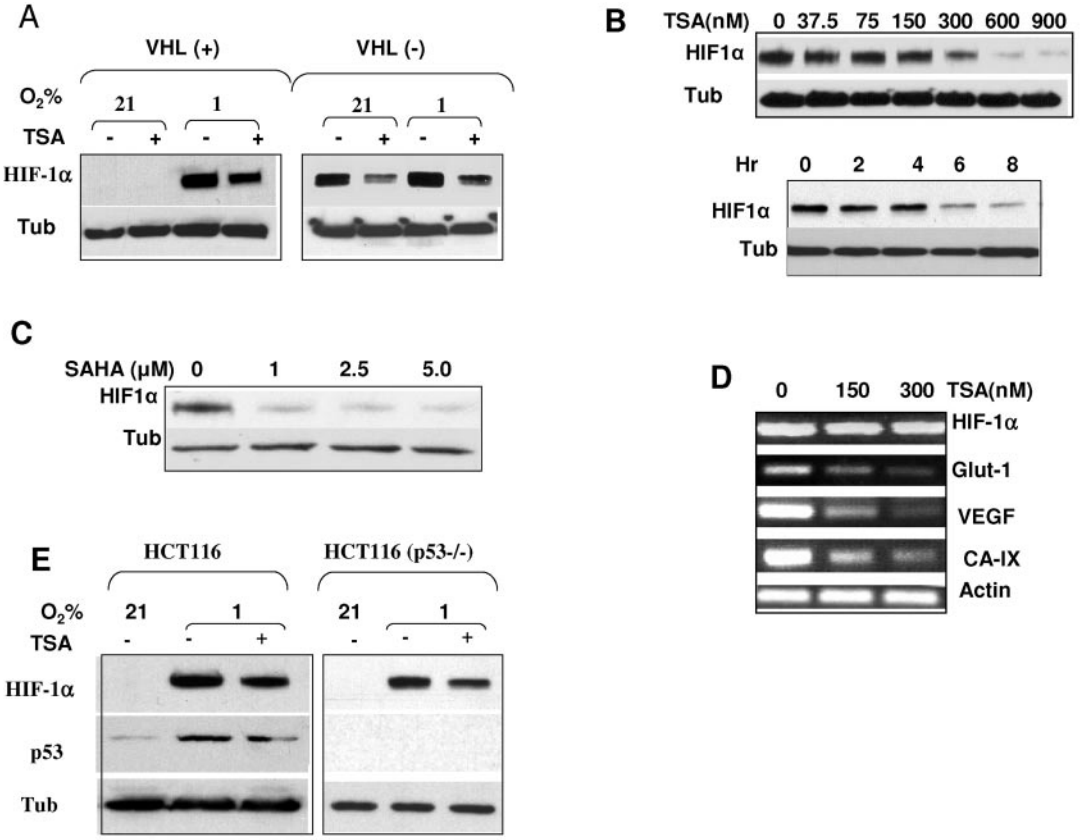


FIG. 2. TSA represses HIF-1α independently of VHL and p53. A. VHL independence. RCC4-VHL$^+$ and -VHL$^-$ cells were treated under normoxia and hypoxia conditions for 8 h in the presence or absence of TSA (300 nM), and cell lysates were analyzed by Western blotting. Tub, tubulin. B. Dose responses and time courses. Normoxic VHL$^-$ cells were exposed to increasing concentrations of TSA for 8 h (upper panels) or to 300 nM of TSA for different time periods (lower panels). C. Effect of SAHA on HIF-1α expression. VHL$^-$ cells were exposed to increasing concentrations of SAHA for 8 h. D. TSA suppresses gene expression in VHL$^-$ cells. VHL$^-$ cells were exposed to TSA for 8 h, and total RNA was analyzed by RT-PCR. E. p53 independence. HCT116 p53$^+$ or p53$^-$ cells were exposed to TSA (300 nM) for 8 h, and cell lysates were analyzed for HIF-1α and p53 expression. VEGF, vascular endothelial growth factor.

was GAACCTGATGCTTTAACT and the reverse primer was CAACTGATC GAAGGAACG; for CAIX (200 bp), the forward primer was CTGTCACTG CTGCTTCTGAT and the reverse primer was TCCTCTCCAGGTAGATCCTC; and for Glut1 (401 bp), the forward primer was CCATGGAGCCCAGCAGCA AG and the reverse primer was GCAGTACACACCGATGATGA.

## RESULTS

**Effect of HDACIs on HIF-1α response to hypoxia.** We studied the effect of HDAC type I and II inhibitors on HIF-1α expression in response to hypoxia in several tumor-derived cell lines. In U-87 cells, TSA produced a dose- and time-dependent inhibition of HIF-1α levels stimulated by hypoxia. Figures 1A and B show that the inhibitory effect of TSA appears at about 300 nM and is evident already at 4 h after initiation of treatment. Similar inhibition was observed with SAHA and to a lesser extent with sodium butyrate (not shown), another type I and II deacetylase inhibitor. The inhibitory effect of TSA on HIF-1α expression was reproduced with variable efficiency in a number of different tumor cell lines, as shown in Fig. 1C. Of importance, at the doses and times utilized these inhibitors did not induce any apoptotic changes and cell viability was well preserved. The inhibition by TSA was equally effective with hypoxia as well as with the hypoxic mimics desferrioxamine, an iron chelator, the transition metal cobalt, and the oxoglutarate analog dimethyloxalylglycine (not shown). Most importantly, the suppressive effect of TSA on HIF-1α resulted in significant inhibition of hypoxia-responsive genes such as vascular endothelial growth factor, Glut1, and CAIX, while no effect was observed in the levels of HIF-1α mRNA transcripts (Fig. 1D).

**TSA-mediated repression of HIF-1α is independent of VHL and p53 but is mediated by the proteasome system.** To further investigate the underlying mechanisms of TSA-mediated repression of HIF-1α, we tested whether this effect required a functional VHL protein and an active proteasome system. TSA treatment of the VHL-deficient RCC4 cells, which express HIF-1α constitutively, revealed that TSA destabilized HIF-1α independently of VHL function and under both normoxic and hypoxic conditions (Fig. 2A). Similar results were obtained with C2 cells, another VHL-deficient renal carcinoma cell line (not shown). Dose response and time course analysis indicated that in VHL-deficient cells, HIF-1α was inhibited by TSA concentrations of 300 to 600 nM and that at this concentration it was effective at about 6 h of exposure (Fig. 2B). The inhibitory effect of TSA on VHL$^-$ cells was reproduced by SAHA (Fig. 2C), another HDACI currently in use in clinical trials, at concentrations that are within the range of

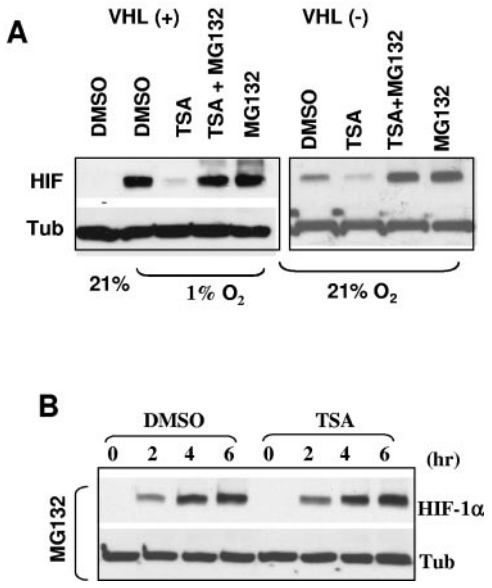


FIG. 3. TSA-induced degradation of HIF-1α is mediated by the proteasome system. A. Proteasome inhibitors reverse TSA effect. RCC4 VHL$^+$ or VHL$^-$ cells were exposed to TSA (300 nM) in the presence or absence of MG132 (5 μM) for 8 h, and cell lysates were analyzed by Western blotting. DMSO, dimethyl sulfoxide. B. TSA does not affect HIF-1α translation. Normoxic U-87 cells were exposed to MG132 (5 μM) in the presence or absence of TSA, and HIF-1α accumulation was measured at 2, 4, and 6 h. Tub, tubulin.

those obtained in vivo (11). As seen with VHL$^+$ cells, treatment of VHL$^-$ cells with TSA suppressed the expression of HIF-1α target genes (Fig. 2D). Since p53 has also been implicated in the degradation of HIF-1α (44), we tested the role of p53 by utilizing HCT116 cells, a p53-deficient cell line, and its p53-reconstituted control (HCT116-p53). Figure 2E shows that although TSA was not as effective in HTC116 cells as in other cell lines, the presence or absence of p53 did not affect the response of HIF-1α to TSA.

We then explored the role of the proteasome system in the TSA-induced repression of HIF-1α by use of the proteasome inhibitors MG132 and epoxomicin (15). Figure 3A shows that use of MG132 completely reversed the effects of TSA in VHL$^+$ and VHL$^-$ cells. Furthermore, this effect was reproduced by epoxomicin, a highly specific proteasome inhibitor, but not by Z-VAD-FMK (a caspase inhibitor) or Z-Leu-Leu-CHO (a calpain inhibitor) (not shown). The finding that proteasome inhibitors completely restored the HIF-1α levels indicated that TSA affected HIF-1α degradation through the proteasome system. The effect of TSA on HIF-1α translation was investigated by measuring the rate of HIF-1α accumulation in normoxic cells exposed to MG132. Figure 3B shows that TSA did not affect the progressive accumulation of HIF-1α in normoxic U-87 cells exposed to MG132 for up to 6 h. Similarly, TSA did not affect the accumulation of other short-lived proteins such as cyclin D1 or p53 (not shown), confirming that TSA is not a general inhibitor of translation and suppresses HIF-1α by en-

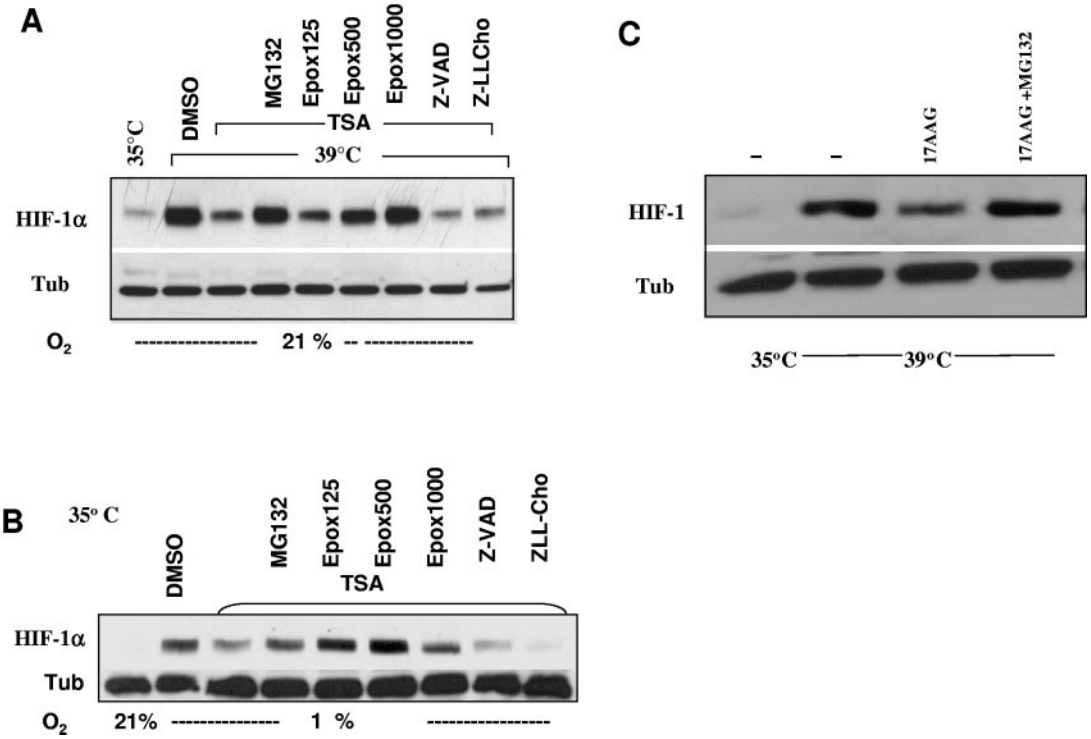


FIG. 4. TSA-induced HIF-1α degradation is independent of ubiquitination. A. TSA degrades HIF-1α in E1-deficient cells. Normoxic Ts20 cells, expressing a temperature-sensitive E1 enzyme, were exposed to 39°C for 8 h in the presence or absence of TSA and in the presence of the proteasome inhibitor MG132 (5 μM) or the indicated concentrations of epoxomicin (Epox), the caspase inhibitor Z-VAD-FMK (50 μM), or the calpain inhibitor Z-Leu-Leu-Cho (40 μM). HIF-1α expression was analyzed by Western blotting. Tub, tubulin. B. E1-competent Ts20 cells respond to TSA. Ts20 cells cultured at 35°C were exposed to hypoxia (1% $O_2$) in the presence of TSA and the inhibitors described for panel A. C. 17AAG degrades HIF-1α in Ts20 cells. Normoxic Ts20 cells were exposed to 39°C for 8 h in the presence or absence of 17AAG (1 μM) and in the presence or absence of the proteasome inhibitor MG132 (5 μM).

hancing its degradation through a mechanism that is independent of hypoxia and VHL but requires the activity of the proteasome system.

**Role of ubiquitin in the TSA-induced degradation of HIF-1α.** Normoxic HIF-1α degradation requires VHL-mediated ubiquitination to undergo proteasomal degradation. To examine whether TSA-induced HIF-1α degradation depends on the ubiquitin system we utilized Ts20 cells, which lack ubiquitination due to the presence of a temperature-sensitive ubiquitin-activating enzyme, E1 (8). In these cells, elevation of the temperature to 39°C leads to inhibition of ubiquitination and a fast accumulation of HIF-1α, even under normoxic conditions (45). Figure 4A shows that in Ts20 cells cultured at 39°C, TSA treatment produced a significant inhibition of HIF-1α protein. Most importantly, MG132 and epoxomicin completely reversed this effect, while caspase or calpain inhibitors did not. Ts20 cells cultured at 35°C and exposed to hypoxia had a similar response to TSA that was also reversed by MG132 and epoxomicin treatment (Fig. 4B). The lack of HIF-1α ubiquitination following combined TSA and MG132 treatment was confirmed by antiubiquitin Western blot analysis of immunoprecipitated HIF-1α from RCC4 cells (not shown). These results indicated that TSA-induced degradation of HIF-1α is ubiquitination independent and mediated by the proteasome system. The role of the ubiquitin system in the non-oxygen-related degradation of HIF-1α was further explored by the use of 17AAG, a HSP90 inhibitor known to suppress HIF-1α expression (20, 27). As shown in Fig. 4C, treatment of Ts20 cells at 39°C with 17AAG suppressed HIF-1α expression and this effect was reversed by MG132, indicating that the ubiquitin system is not an absolute requirement for the proteasomal degradation of HIF-1α.

**The ODD of HIF-1α is sufficient and necessary for TSA-mediated degradation.** To investigate the role of the oxygen-dependent ODD in TSA-mediated degradation of HIF-1α, a plasmid expressing the HIF-ODD (aa 393 to 580) fused to a *myc* tag and a nuclear localization signal was transfected into HT1080 cells and exposed to TSA. Figure 5A (upper panels) shows that treatment with TSA suppresses the expression of HIF-ODD under both normoxic and hypoxic conditions. A substitution of lysine-532 to arginine (K532R) at the putative acetylation site described by Jeong et al. failed to affect the level of expression of the ODD, its response to hypoxia, or the effect of TSA. Conversely, a construct resulting from deletion of the HIF-ODD (ΔODD; aa 392 to 575) was not significantly affected by TSA whereas a full-length HIF-1α (Fig. 5A; myc-HIF) and the endogenous HIF-1α (Fig. 5A; HIF) were both reduced by TSA treatment. Moreover, a full-length HIF with mutations in both proline hydroxylation sites (HIF-P402A/P564G) that is not degraded under normoxia conditions was dose-dependently affected by TSA (Fig. 5B). These data indicate that TSA-induced HIF-1α degradation requires the ODD but is not dependent on K532 acetylation and does not require prolyl hydroxylation.

**TSA enhances the interaction between HIF-1α and HSP70.** Ubiquitin-independent degradation of proteins by the proteasome system has been described for several proteins, including ornithine decarboxylase, the cyclin-dependent kinase inhibitor $p21^{cip1}$, and p53 (33). Our finding that the ODD is involved in the TSA-mediated degradation of HIF-1α and the previous

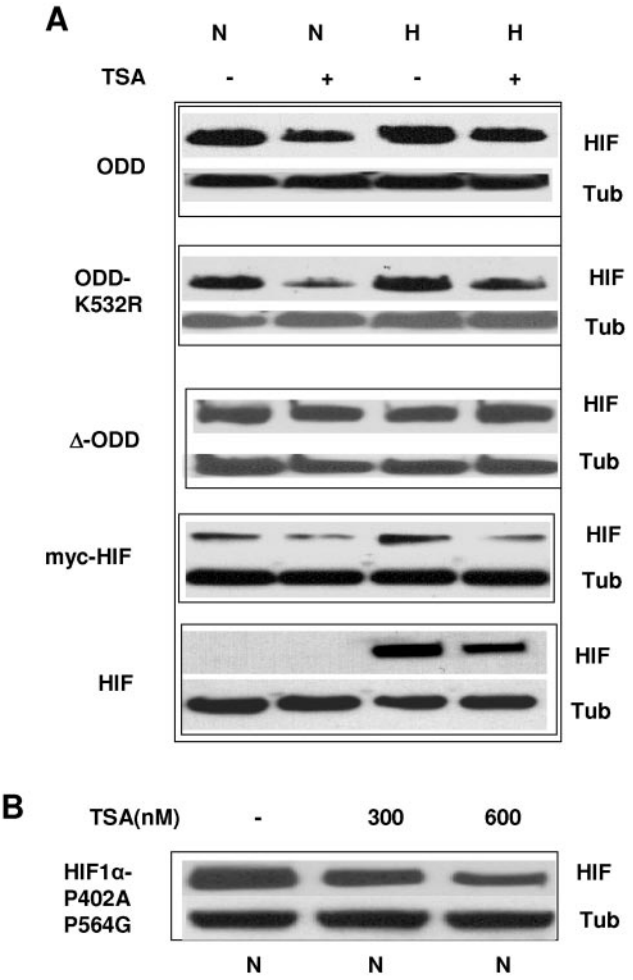


FIG. 5. HIF-1α-ODD is sufficient and necessary for TSA-induced degradation. A. ODD determines response to TSA independently of K532 acetylation. HT1080 cells were transfected with plasmids containing the ODD (top pair of panels), a K532R ODD mutant (second pair of panels), an ODD-deleted HIF-1α construct (ΔODD; third pair of panels), or a whole HIF-1α construct (myc-HIF; fourth pair of panels), and the cells were exposed to TSA (300 nM) for 8 h under normoxic (N) or hypoxic (H) conditions. The fifth pair of panels (HIF) presents the endogenous HIF-1α protein. B. The TSA effect is independent of prolyl hydroxylation. A HIF-1α construct containing P402A and P564G mutations was transfected into HT1080 cells and exposed to increasing concentrations of TSA for 8 h under normoxia conditions. Cell lysates were analyzed by Western blotting.

observation of the interaction between HSP70 and HIF-ODD (55) prompted us to study whether TSA also affects this interaction. Immunoprecipitation studies showed that TSA treatment enhances the interaction between HIF-1α and HSP70 (Fig. 6A and B). This interaction was TSA dose dependent and was observed using either anti-HIF-1α (Fig. 6A) or anti-HSP70 (Fig. 6B) as the precipitating antibody. Of interest, TSA treatment increased the amount of insoluble HIF-1α protein recovered from cell lysate pellets after centrifugation and this effect was more evident in the combined TSA- and MG132-treated cells (Fig. 6C). HDACIs have been implicated in the proteasomal degradation of several proteins by inducing hyperacetylation and inhibition of function of the chaperone HSP90 in a

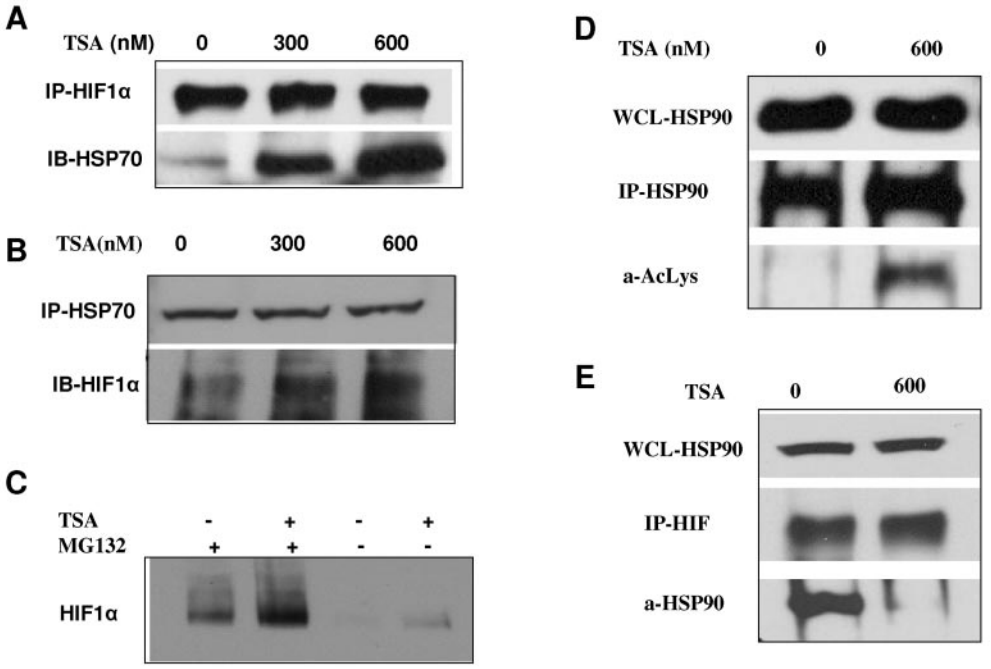


FIG. 6. TSA enhances the interaction between HIF-1α and HSP70. A. HIF-1α interacts with HSP70. RCC4-VHL$^-$ cells were treated with increasing concentrations of TSA for 4 h, and cell lysates were immunoprecipitated (IP) with anti-HIF-1α monoclonal antibodies. The precipitate was immunoblotted with anti-HIF-1α and anti-HSP70. B. HSP70 interacts with HIF-1a. Cells were treated as described for panel A, but lysates were precipitated with anti-HSP-70 monoclonal antibodies. C. TSA treatment increases HIF-1α in lysate pellets. VHL$^-$ cells were treated for 6 h in the presence or absence of MG132 and TSA. The cell lysates were centrifuged, and the cell pellets were resuspended in loading buffer and analyzed by Western blotting. D. TSA treatment enhances HSP90 acetylation. RCC4-VHL$^-$ cells were treated with TSA (600 nM) for 6 h followed by immunoprecipitation with anti-HSP90 antibodies. Whole-cell lysates and immunoprecipitation results were analyzed by Western blotting utilizing anti-HSP90 and anti-acetylated lysine (a-AcLys) antibodies. E. TSA treatment decreases the interaction between HSP90 and HIF-1α. RCC4-VHL$^-$ cells were incubated with TSA (600 nM) for 6 h followed by immunoprecipitation with anti-HIF-1α antibodies. The immunoprecipitation results was analyzed by Western blotting using anti-HIF-1α and anti-HSP90 antibodies.

process that appears to be mediated by HDAC-6 (31). As HIF-1α is a client protein of HSP90, we investigated the effect of HSP90 acetylation and the possible role of HDAC-6 in the degradation of HIF-1α by TSA. As shown in Fig. 6D, TSA treatment induced hyperacetylation of HSP90 and decreased the interactions between HIF-1α and HSP90. To study the role of HDAC-6 we utilized cell lines engineered to have increased or suppressed HDAC-6 levels. The A549–HDAC-6 cells express high HDAC-6 activity and have low levels of acetylated HSP90, while the 293-KD (knockdown) cells express low HDAC-6 activity and have hyperacetylated HSP90 (31). Figure 7A shows that overexpression of HDAC-6 results in higher hypoxic levels of HIF-1α and a relative resistance to TSA treatment. Conversely, HDAC-6 knockdown cells showed inhibition on HIF-1α protein in response to hypoxia (Fig. 7B) and suppression of hypoxia-responsive genes (Fig. 7C). These results indicate that HDAC-6 is involved in the TSA effect on HIF-1α by affecting the acetylation status of HSP90, thus inhibiting the function of the HSP70/HSP90 axis, as schematically represented in Fig. 8.

## DISCUSSION

Based on their potent proapoptotic and antiangiogenic effect, deacetylase inhibitors are being tested as potentially important new chemotherapeutic agents for the treatment of solid tumors (reviewed in reference 11). Suppression of the HIF-1α response appears to play a key role in their antiangiogenic effects (29, 41, 51). Under normoxic conditions HIF-1α is rapidly degraded by the ubiquitin-proteasome system in a process that depends on the interaction between the HIF-1α–ODD and the VHL protein that acts as an E3 ligase. Thus, VHL-deficient cells constitutively express high levels of HIF-1α protein and HIF-controlled genes (30). Our initial experiments confirmed that TSA, SAHA, and, to a lesser extent, sodium butyrate suppressed HIF-1α expression in response to hypoxia and hypoxia mimics at doses that did not affect cell viability. Most notably, these inhibitors were equally effective in VHL$^-$ and VHL$^+$ cells but utilized the proteasome degradation system. The finding that proteasome inhibitors completely reversed the TSA effect indicates that HIF-1α degradation is the primary mechanism involved in TSA action. The lack of effect of TSA on HIF-1α translation was confirmed by the observation that TSA did not affect the rate of accumulation of HIF-1α protein in normoxic cells incubated with MG132 and that it also did not affect the rate of translation of other short-lived proteins.

Acetylation of the ε amino group of lysyl residues has emerged as an important posttranslational modification regulating protein functions. In the case of transcription factors, acetylation can affect DNA binding, subcellular localization, transcriptional activity, and protein survival (24). The degree

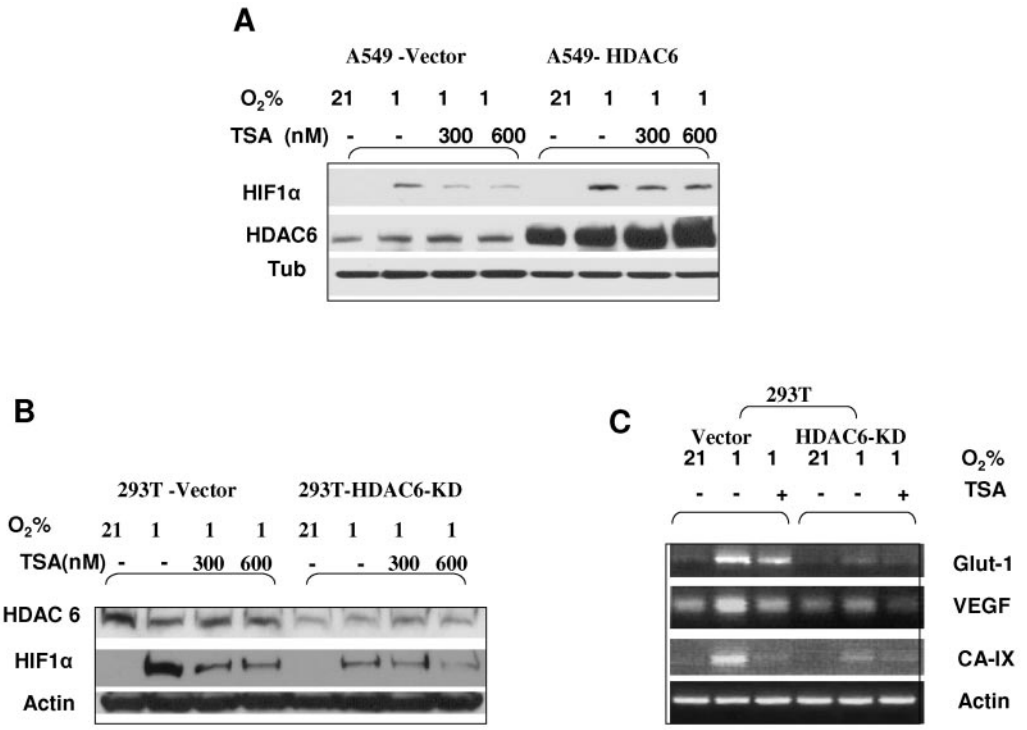


FIG. 7. HDAC-6 is involved in the TSA-induced degradation of HIF-1α. A. Overexpression of HDAC-6 enhances HIF-1α levels in response to hypoxia. Control A549 (vector) and overexpressing HDAC-6 cells were subjected to 1% $O_2$ for 8 h at the indicated concentrations of TSA. Cell lysates were analyzed by Western blotting using anti-HIF-1α, anti-HDAC-6, and anti-tubulin (Tub) antibodies. B. Knockdown of HDAC-6 reduces HIF-1α levels in response to hypoxia. Control 293T cells (Vector) and HDAC-6 knockdown cells (KD) were treated and analyzed by Western blotting as described for panel A. C. Knockdown of HDAC-6 suppresses hypoxia-inducible gene expression. Vector and knockdown cells were treated as described for panel A, and total RNA was analyzed by RT-PCR. VEGF, vascular endothelial growth factor.

of acetylation of a given protein depends on the dynamic balance of the activity of specific acetylase and deacetylase enzymes. Although HDACs are mostly known to deacetylate histones, they also interact with other proteins, notably transcription factors, to affect their functions. For example, HDACI-mediated deacetylation of p53 promotes its ubiquitination and degradation (34). Recently, Jeong and coworkers reported that HIF-1α protein was acetylated at the K532 residue by ARD1 (Arrest-Defective-1), promoting its interaction with VHL and thus enhancing its ubiquitination and degradation (23). In *Saccharomyces cerevisiae*, ARD1 acts in concert with NAT-1 to form an active complex with $N^{\alpha}$-terminal acetylation activity (43), while in mammals, ARD1 function has not been well characterized (1). Our findings that the effect of TSA was VHL independent and that mutation of the putative HIF-1α K532 acetylation site did not affect TSA actions are strong evidence against the hypothesis that HDACIs act through enhancing VHL/HIF-1α interactions (23, 29). Further evidence against the involvement of ARD1 in HIF-1α degradation comes from recent reports that ARD1 does not acetylate HIF-1α (2) and that overexpression or suppression of ARD1 had no effect on HIF-1α levels (2, 5, 13).

The observation that TSA was able to suppress HIF-1α in Ts20 cells containing a temperature-sensitive E1 enzyme (Fig. 4) indicates that ubiquitination of HIF-1α is not a prerequisite for its proteasomal degradation. Non-ubiquitin-mediated proteasomal degradation has been described for several proteins, including ODC, $p21^{cip1}$, c-Jun, and p53 (33). In the case of p53, degradation is independent of MDM2 and is regulated by the activity of the enzyme NAD(P)H-quinone oxidoreductase (NQO-1) (3). The ubiquitin-independent degradation of proteins is mediated by the free 20S proteasome that normally degrades naturally unfolded or damaged proteins. Targeting of a substrate to the 20S proteasome can be brought about by accessory molecules or by sequences within the substrate itself (33). Thus, Tax increases the binding of IκBα to the B7 subunit of the 20S proteasome, while $p21^{cip1}$ degradation is mediated by the binding of the terminus of the protein to the C8-α subunit of the 20S proteasome (32).

In the case of HIF-1α, our results suggest that the ODD sequences are necessary for the targeting of HIF-1α to the proteasome following TSA treatment. Of interest, HIF-1α-ODD has been found to interact with PSMA7, one of the α-type subunits of the 20S proteasome-core complex, and overexpression of PSMA7 was described as enhancing HIF-1α degradation (7). Hydroxylation of prolyl residues in the ODD targets HIF-1α for ubiquitination and proteasomal degradation. However, the effect of TSA is independent of hypoxia or the activity of the prolyl hydroxylase enzymes (Fig. 5). Our immunoprecipitation studies demonstrated that TSA treatment enhanced the interaction between HIF-1α and HSP70, a family member of the chaperone class of proteins. Of importance, heat shock proteins are known to interact with HIF-1α and to affect its survival. While HSP90 interacts with the

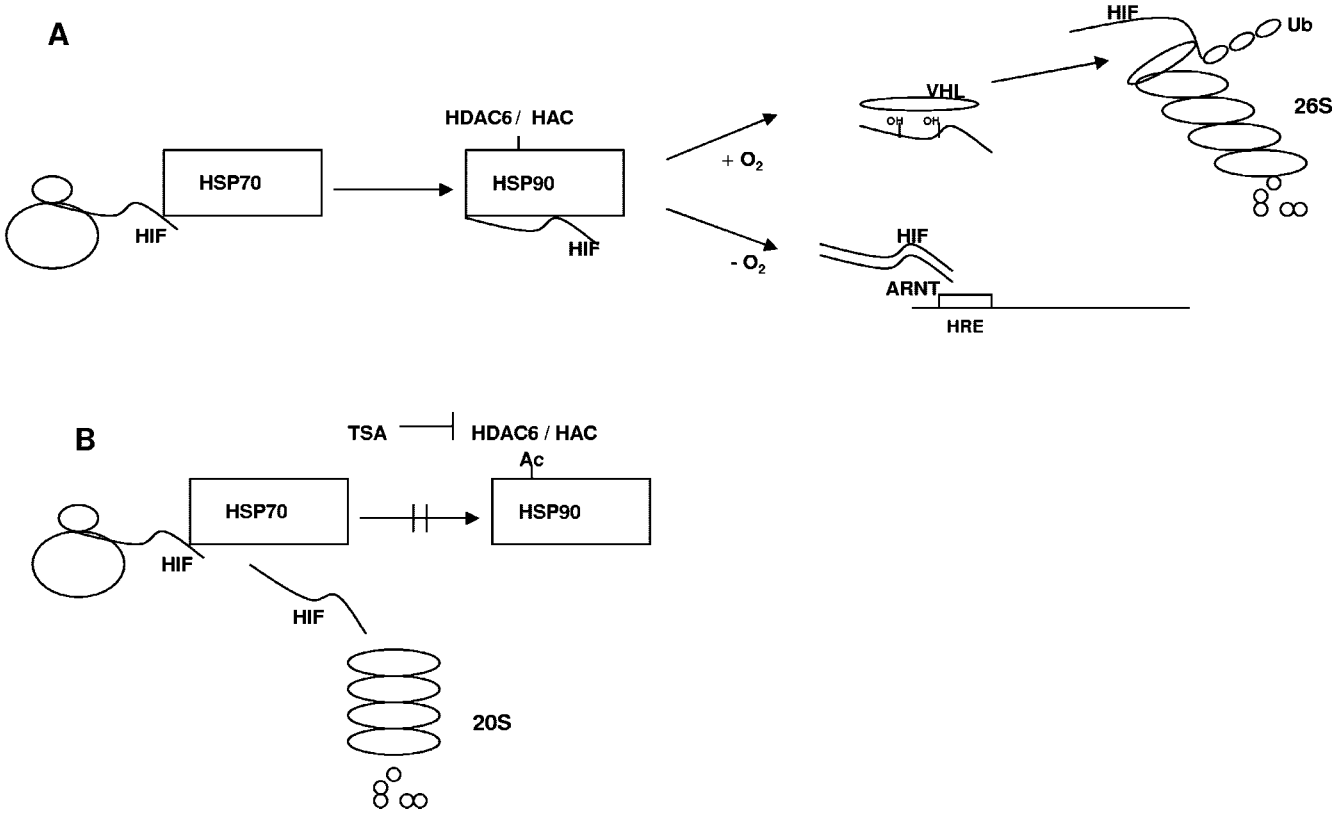


FIG. 8. Schematic representation of the mechanisms involved in HIF-1α degradation following HDACI treatment. A. In the absence of HDACIs, newly synthesized HIF-1α molecules interact with its chaperones HSP70 and HSP90 to complete its maturation. Under normoxic conditions ($+O_2$), the mature protein is hydroxylated, ubiquitinated, and degraded by the 26S proteasome, while under hypoxia conditions ($-O_2$), the protein survives, interacts with ARNT, and binds hypoxia response element (HRE) sequences to initiate transcription. B. During HDACI treatment, HDAC-6 inhibition results in hyperacetylation of HSP90, accumulation of immature HIF-1α protein/HSP70 complex, and degradation of HIF-1α by the 20S proteasome.

*p*-aminosalicylic acid domain of HIF-1α (20, 28), HSP70 interacts with the ODD (55). Treatment with the HSP90 inhibitor geldanamycin or 17AAG produces a marked suppression of HIF-1α expression by affecting its folding and maturation and inducing its degradation (20, 27).

Recently, evidence was presented that HSP90 chaperone activity is regulated by its reversible acetylation controlled by HDAC-6 (4, 31, 42). Treatment of cells with HDACIs or suppression of HDAC-6 expression produced hyperacetylation of HSP90 and inhibition of its chaperone function, with consequent accelerated degradation of client proteins (16, 31, 42). Nascent polypeptides at the ribosome can expose hydrophobic amino acids residues that need to be stabilized cotranslationally to prevent abnormal folding and protein deposition. The HSP70 protein complexes, acting upstream of HSP90, transiently hold nascent peptides, preventing irreversible aggregation and catalyzing refolding in an ATP- and chaperone-dependent process. However, when the proper folding is not achieved or the HSP70-HSP90 chaperone axis cycle is interrupted, the unfolded proteins are targeted for degradation (39, 50). This process may be mediated by ubiquitination, where HSP70 requires its association with Bag 1 and CHIP, or by ubiquitin-independent degradation, where the substrates are targeted directly to the 20S proteasome (38).

The proteolytic activity of the proteasome is severely impaired in the presence of highly aggregated proteins, and the presence of HSP70 appears to facilitate the proteolytic process. Our finding that TSA treatment enhanced HIF-1α/HSP70 interactions and increased the insoluble HIF-1α fraction (Fig. 6) suggests that HSP70 is involved in the triage of HIF-1α to the 20S proteasome. Whether this is a direct process or requires the presence of other targeting proteins is under investigation. Similarly, it is unclear whether direct acetylation of HSP70 or HIF-1α is involved. Though we utilized several anti-acetyllysine antibodies and failed to demonstrate acetylation on either protein, these results do not completely rule out this possibility. More likely, however, HDAC inhibitors affect the HSP70/HSP90 axis by determining the degree of HSP90 acetylation and its ability to interact with HIF-1α (Fig. 6C and 6D). Although the exact mechanisms by which hyperacetylation of HSP90 impairs its function remain to be established, recent findings suggest that it decreases ATP binding and thereby reduces its interactions with p23. The acetylation status of HSP90 appears mediated by the dynamic equilibrium between HDAC-6 and still-unidentified acetyltransferases. Studies by Kovacs et al. (31) and Bali et al. (4) conducted using HDAC-6 suppression showed that depletion of HDAC-6 produced hyperacetylation of HSP90, reduced the chaperone association

with client proteins, i.e., bcr-abl and glucocorticoid receptor, and induced either ubiquitin-dependent degradation or abnormal function of the proteins. Of interest, our results indicate that ubiquitination of the client protein is not an absolute requisite for its degradation. Indeed, ubiquitin-independent HIF-1α degradation was also observed by using the HSP90 inhibitor 17AAG (Fig. 4C). Whether this process is peculiar to HIF-1α or is part of a general mechanism of protein degradation is still unclear.

In summary, our experimental findings indicate that HDACIs induce ubiquitin-independent degradation of HIF-1α. The most likely mechanism of action implicates their effects on the HSP70-HSP90 axis, preventing the proper folding and maturation of the HIF-1α protein and thus promoting its degradation by the proteasome in a ubiquitin-independent process, as schematically displayed in Fig. 8. Of importance, the finding that HIF-1α degradation induced by HDACIs is independent of the presence of functional VHL and p53 expands the application of these drugs to p53- and VHL-deficient tumors.

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