Spermidine/Spermine N¹-Acetyltransferase-1 Binds to Hypoxia-inducible Factor-1α (HIF-1α) and RACK1 and Promotes Ubiquitination and Degradation of HIF-1α

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Hypoxia-inducible factor-1 (HIF-1) is a master regulator of oxygen homeostasis that controls the expression of genes encoding proteins that play key roles in angiogenesis, erythropoiesis, and glucose/energy metabolism. The stability of the HIF-1α subunit is regulated by ubiquitination and proteasomal degradation. In aerobic cells, O2-dependent prolyl hydroxylation of HIF-1α is required for binding of the von Hippel-Lindau tumor suppressor protein VHL, which then recruits the Elongin C ubiquitin-ligase complex. SSAT2 (spermidine/spermine N-acetyltransferase-2) binds to HIF-1α and promotes its ubiquitination/degradation by stabilizing the interaction of VHL and Elongin C. Treatment of cells with heat shock protein HSP90 inhibitors induces the degradation of HIF-1α even under hypoxic conditions. HSP90 competes with RACK1 for binding to HIF-1α, and HSP90 inhibition leads to increased binding of RACK1, which recruits the Elongin C ubiquitin-ligase complex to HIF-1α in an O2-independent manner. In this work, we demonstrate that SSAT1, which shares 46% amino acid identity with SSAT2, also binds to HIF-1α and promotes its ubiquitination/degradation. However, in contrast to SSAT2, SSAT1 acts by stabilizing the interaction of HIF-1α with RACK1. Thus, the paralogs SSAT1 and SSAT2 play complementary roles in promoting O2-independent and O2-dependent degradation of HIF-1α.

The maintenance of oxygen homeostasis is a critical developmental and physiological imperative. Hypoxia-inducible factor-1 (HIF-1) plays a major role in regulating gene transcription in response to changes in O2 availability in all metazoan species. HIF-1 regulates the transcription of hundreds of genes in hypoxic human cells (1, 2). The products of these genes include proteins such as erythropoietin, vascular endothelial growth factor, GLUT1 (glucose transporter-1), and lactate dehydrogenase A, which control erythropoiesis, angiogenesis, glucose uptake, and glycolysis, respectively. These are adaptive physiological responses to hypoxia that serve either to increase O2 delivery to cells (3, 4) or to allow cells to survive under conditions of reduced O2 availability by activating alternative metabolic pathways that do not require O2 and that reduce the formation of reactive oxygen species (5–7).

HIF-1 is a heterodimeric protein that consists of a constitutively expressed HIF-1β subunit and a highly regulated HIF-1α subunit (8, 9). The N-terminal half of HIF-1α and HIF-1β consists of basic helix-loop-helix and PAS (PER-ARNT-SIM homology) domains, which are required for dimerization and DNA binding (9, 10), whereas the C-terminal half of HIF-1α consists of regulatory domains that control protein stability and transactivation function (10–13).

Three major pathways for regulation of HIF-1α protein half-life have been delineated, two of which involve ubiquitination and proteasomal degradation. The first pathway described is O2-dependent and involves binding of HIF-1α to the von Hippel-Lindau tumor suppressor protein VHL, which recruits an E3 ubiquitin-protein isopeptide ligase complex that includes Elongin C, Elongin B, cullin-2, and RBX1 (ring box protein-1) (14, 15). Binding of VHL to HIF-1α is dependent upon the hydroxylation of proline residue(s) 402 and/or 564 by the HIF-1α prolyl hydroxylase PHD2 (prolyl hydroxylase domain protein-2) (3, 16, 17). HIF-1α prolyl hydroxylases utilize O2 as a substrate, and their catalytic activity is inhibited under hypoxic conditions (18–20). A second pathway that is independent of prolyl hydroxylation and VHL has been described in which heat shock protein HSP90 inhibitors, such as geldanamycin and 17-allylamino geldanamycin (17-AAG), induce the proteasomal degradation of HIF-1α (21, 22). The degradation of HIF-1α by HSP90 inhibitors contributes to the anti-angiogenic and anti-cancer activity of these compounds in xenograft models, and 17-AAG is currently being evaluated in clinical trials. Loss of HSP90 binding to HIF-1α in cancer cells treated with 17-AAG leads to increased binding of RACK1 (receptor for activated C kinase-1), which recruits Elongin C and its E3 ubiquitin-protein isopeptide ligase complex to HIF-1α in an O2-independent manner (23). Thus, the Elongin C complex can be recruited to HIF-1α either by O2-dependent binding of VHL or by O2-independent, 17-AAG-inducible binding of RACK1. In both cases,...
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FIGURE 1. SSAT1 inhibits HIF-1α through protein/protein interaction. A, human 293 cells were cotransfected with expression vector encoding FLAG-HIF-1α, V5-SSAT2, V5-SSAT1, and/or empty vector to maintain a constant amount of transfected DNA. After 24 h, whole cell lysates (WCL) were prepared, and immunoblot assays were performed with antibodies against FLAG, V5, or β-actin using WCL directly. SSAT2 (spermidine/spermine N-acetyltransferase) interacts with both HIF-1α and PHD2 and is required for efficient prolyl hydroxylation (29), SSAT1, FLAG-HIF-1α, or empty vector (EV) for 24 h. The ratio of firefly to Renilla luciferase activity was determined. The results were normalized to those from cells transfected with empty vector (Luciferase Activity). The means ± S.D. obtained from three independent transfections are shown, *p < 0.05. C, to identify HIF-1α sequences required for binding to SSAT1 in vitro, GST and GST fusion proteins containing the indicated residues of HIF-1α were purified from bacteria, incubated with in vitro translated, 35S-labeled SSAT1, captured on glutathione-Sepharose beads, and analyzed by SDS-PAGE and autoradiography (upper panel). T/I, 10% of the total input of SSAT1 was analyzed by direct loading onto the gel. GST proteins were analyzed by SDS-PAGE and immunoblot assay using anti-GST antibody to quantify input levels (lower panels). D, cells were transfected with expression vector encoding FLAG-HIF-1α or V5-SSAT1 or empty vector and treated with 10 μM MG132 for 6 h. WCL were prepared, and immunoprecipitation was performed using anti-V5 antibody-conjugated agarose beads. Immunoblot assays were performed with antibody against FLAG (upper panels), V5 (middle panel), or β-actin (lower panel) using 80 μg of WCL directly (right panels) or after immunoprecipitation of 500 μg of WCL (left panel). Con, control.

the result is ubiquitination and proteosomal degradation of HIF-1α. A third pathway has been proposed to account for the degradation of HIF-1α in cells treated with immunophilins or histone deacetylase inhibitors based on recent studies suggesting that these agents induce degradation by an O2-independent mechanism that is also ubiquitin-independent (24–26).

The rapid degradation of HIF-1α in response to re-oxygenation (9, 27, 28) or treatment with HSP90 inhibitors (21, 22) implies the existence in human cells of precise mechanisms for rapidly modulating HIF-1α protein levels. The precision and speed of these responses appear to be due in part to the presence of multiple regulatory proteins that facilitate the interaction of HIF-1α with the hydroxylation and ubiquitination machinery. OS-9 (osteosarcoma protein-9) binds to both HIF-1α and PHD2 and is required for efficient prolyl hydroxylation (29). SSAT2 (spermidine/spermine N-acetyltransferase) binds to HIF-1α, VHL, and Elongin C and is required for efficient VHL-mediated ubiquitination (30). Here, we report that the protein SSAT1 interacts with both HIF-1α and RACK1 and is required for RACK1-mediated ubiquitination and degradation of HIF-1α in cells treated with 17-AAG.

EXPERIMENTAL PROCEDURES

Tissue Culture—Human 293 and 293T cells were cultured as described (31). Cells were maintained at 37 °C in a 5% CO2 and 95% air incubator. For hypoxic exposures, cells were placed in a Billups-Rothenberg modular incubator chamber that was flushed with 1% O2, 5% CO2, and balance N2; sealed; and incubated at 37 °C.

In Vitro Binding Assay Using Glutathione S-Transferase (GST) Fusion Proteins—Escherichia coli BL21-Gold(DE3)pLysS cells (Stratagene) were transformed with pGEK expression vectors and treated for 4 h with 0.5 mM isopropyl β-thio-galactopyranoside. Cell lysates were applied to glutathione-Sepharose 4B beads (GE Healthcare). GST fusion proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). [35S]Methionine-labeled proteins were generated in reticulocyte lysates using the TNT T7 coupled transcription/translation system (Promega Corp.). Ten μl of in vitro translated, 35S-labeled protein was mixed with 4 μg of GST, GST-HIF-1α (30, 32), GST-RACK1 (23), or GST-Elongin C (30) fusion protein in PBS-T binding buffer (Dulbecco’s phosphate-buffered saline (pH 7.4) and 0.1% Tween 20) at 4 °C for 2 h, followed by the addition of 20 μl of glutathione-Sepharose 4B beads. After 30 min of mixing, the beads were washed with PBS-T binding buffer. Proteins were eluted in Laemmli sample buffer and analyzed by SDS-PAGE, followed by autoradiography using Molecular Imager FX (Bio-Rad).

Construction of Expression Vectors—The open reading frame of SSAT1 was amplified from cDNA prepared from human umbilical vein endothelial cell mRNA using specific primers based on the nucleotide sequence of GenBank™ accession number NM_002970 (supplemental Table S1). The PCR product was inserted into the pcDNA3.1(D)/V5-His-TOPO vector (Invitrogen). The coding sequence of SSAT1 was amplified with primers containing a SalI restriction enzyme site at the 5′-end and blunt 3′-end and ligated into the Sall restriction enzyme site at the 5′-end and blunt 3′-end and ligated into the Sall and EcoRV sites of pGEX-5X-1 (GE Healthcare).

Transfection Assays—293 cells were seeded onto 48-well plates at 4 × 104 cells/well and transfected with plasmid DNA using FuGENE 6 (Roche Applied Science). After 24 h, the cells were exposed to 20 or 1% O2 for 24 h. Cells were lysed, and luciferase activities were determined with a multiwell luminometer using the Dual-Luciferase reporter assay system (Promega Corp.). For p2.1 reporter assay, cells were cotransfected with 6 ng of control reporter pSV-Renilla; 56 ng of HIF-1α reporter p2.1 (31); and expression vector encoding SSAT1, HIF-1α, or FLAG-HIF-1α or empty vector. Unless indicated otherwise, 80 ng of FLAG-HIF-1α, 100 ng of SSAT1, 1 ng of PHD2, or 1 ng of FLAG-HIF-1α(P402A/P564A) expression vector was used. For immunoblot assays, 293 cells were seeded at 1 × 106 cells/6-cm plate. The following
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day, the cells were transfected. After 24 h, the cells were exposed to 20 or 1% O₂ with or without MG132 for 6 h and lysed in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, Complete protease inhibitor mixture (Roche Applied Science), sodium orthovanadate, and sodium fluoride (Sigma).

Immunoprecipitation and Immunoblot Assays—293 cells (3 × 10⁶ cells/10-cm plate) were transfected with plasmids using FuGENE 6. Cells were lysed with PBS-T. Whole cell lysates were immunoprecipitated using immunoglobulin G (Oncogene), anti-hemagglutinin affinity matrix (Roche Applied Science), or anti-FLAG antibody (Sigma), followed by immunoblot assay using antibody against HIF-1α (33), HIF-1β (34), PHD2 (Novus Biologicals), β-actin (Santa Cruz Biotechnology, Inc.), FLAG, ubiquitin, or V5 (Invitrogen).

Short Hairpin RNA (shRNA) Assays—The mammalian expression vector pSR.retro.GFP.Neo.circular.stuffer (OligoEngine) was used for expression of shRNA in 293 cells. The SSAT1 shRNA insert consisted of the 22-nucleotide sequence gcagtaattgaccagta, corresponding to nucleotides 408–429 of SSAT1 mRNA (GenBank™ accession number NM_002970), which was separated by a spacer (tcaagaga) from the reverse complement of the same 22-nucleotide sequence. A scrambled negative control vector constructed using a 19-nucleotide sequence (acgcatgcatgcttt) with no significant homology to any mammalian gene sequence served as a control. Oligonucleotides were annealed and ligated into BglII- and HindIII-digested vector. 293 cells were transfected with expression vector encoding shSNC or the indicated amount of expression vector encoding shSSAT1 or shS1. Immunoblot assays were performed with anti-FLAG, anti-V5, or anti-β-actin antibody. Cells were cotransfected with the control Renilla luciferase reporter pSV-Renilla, the HIF-1-dependent firefly luciferase reporter p2.1, and vector encoding shSNC or shSSAT1. Lysates were prepared 37 h after transfection, and the ratio of firefly to Renilla luciferase activity was determined. The results were normalized to those from cells transfected with shSNC (Luciferase Activity). The means ± S.D. were determined based on three independent transfections. *, p < 0.05.

RESULTS

A yeast two-hybrid screen using amino acid residues 17–299 of human HIF-1α as bait led to the identification of SSAT2 as a human protein that binds to HIF-1α, VHL, and Elongin C and is
required for O₂-dependent ubiquitination and degradation of HIF-1α (30). Because SSAT2 bears 46% amino acid identity to SSAT1, we tested whether the latter protein also regulates HIF-1α protein levels. Human 293 cells were cotransfected with expression vectors encoding FLAG epitope-tagged HIF-1α and either V5 epitope-tagged human SSAT1 or empty vector. Immunoblot assays revealed lower levels of FLAG-HIF-1α in cells coexpressing V5-SSAT1 (Fig. 1A, upper panel). The reduction in FLAG-HIF-1α levels was similar to that obtained by cotransfection of vector encoding V5-SSAT2, even though much higher levels of V5-SSAT2 were achieved compared with V5-SSAT1 (Fig. 1A, middle panel). β-Actin protein levels were not affected by V5-SSAT1 or V5-SSAT2 (Fig. 1A, lower panel).

To demonstrate that this reduction in HIF-1α protein levels was associated with reduced HIF-1α-dependent gene transcription, cells were cotransfected with reporter gene p2.1, in which the expression of firefly luciferase is driven by a hypoxia response element from the ENO1 gene upstream of a basal SV40 promoter (31), and control plasmid pSV-Renilla, in which Renilla luciferase expression is driven by the SV40 promoter alone. Cotransfection of HIF-1α expression vector increased the ratio of firefly to Renilla activity by >4-fold (Fig. 1B). However, this HIF-1α-dependent induction of reporter gene activity was reduced by SSAT2 and eliminated by SSAT1 overexpression.

We next analyzed the binding of in vitro translated SSAT1 to fusion proteins consisting of GST and different HIF-1α protein domains. SSAT1 bound weakly to HIF-1α residues 81–200, which encompass the PAS A-subdomain, and strongly to residues 201–329, which encompass the PAS B-subdomain (Fig. 1C). In contrast, SSAT2 bound strongly to residues 81–200 and did not bind to residues 201–329 (30).

To determine whether HIF-1α and SSAT1 interact in human cells, we cotransfected 293 cells with FLAG-HIF-1α expression vector and either V5-SSAT1 or empty vector. The cells were treated with MG132 to block the proteasomal degradation of HIF-1α. Compared with untreated cells, in which FLAG-HIF-1α levels were markedly lower when V5-SSAT2 was coexpressed (Fig. 1A, first and second lanes), FLAG-HIF-1α levels were equal in MG132-treated cells transfected with V5-SSAT2 or empty vector (Fig. 1D, upper right panel). In cells coexpressing FLAG-HIF-1α and V5-SSAT1, anti-V5 immunoprecipitates were found to contain FLAG-HIF-1α (Fig. 1D, left panel). The results presented in Fig. 1 indicate that SSAT1 interacts with and promotes the degradation of HIF-1α.

To complement the SSAT1 gain-of-function studies described above and to determine whether endogenous SSAT1 is required to maintain low levels of HIF-1α under aerobic conditions, we transfected 293 cells with expression vector encoding an shRNA that targets SSAT1 mRNA for degradation (shSSAT1) or encoding a scrambled negative control shRNA.
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Quantitative real-time reverse transcription-PCR demonstrated that endogenous SSAT1 mRNA levels were significantly reduced in cells transfected with shSSAT1 compared with shSNC-transfected or control non-transfected cells (Fig. 2A). Expression of shSSAT1 also modestly reduced the levels of coexpressed V5-SSAT1 protein (Fig. 2B, middle panel). The reduced expression of V5-SSAT1 in cells expressing shSSAT1 was associated with modestly increased FLAG-HIF-1α levels (Fig. 2B, upper panel). In contrast, β-actin protein levels were not affected by expression of V5-SSAT1 or shSSAT1 (Fig. 2B, lower panel). Notably, transfection of expression vector encoding shSSAT1 increased the endogenous HIF-1α protein levels in a dose-dependent manner but did not affect the levels of HIF-1β or β-actin (Fig. 2C). In cells cotransfected with the HIF-1-dependent p2.1 and control pSV-Renilla reporter plasmids, shSSAT1 expression increased HIF-1-dependent luciferase activity by 2.5-fold relative to cells expressing shSNC (Fig. 2D). Compared with control non-transfected cells, expression of GLUT1 mRNA, which is encoded by a HIF-1 target gene, was significantly increased in cells expressing shSSAT1, but not in cells expressing shSNC (Fig. 2E). Thus, the gain-of-function (Fig. 1) and loss-of-function (Fig. 2) studies demonstrated that SSAT1 is a negative regulator of HIF-1α protein levels and HIF-1-dependent transcription in aerobic cells.

Our recent study of SSAT2 revealed that the protein is required for VHL-dependent ubiquitination of HIF-1α in well oxygenated cells (30), which is triggered by the PHD2-dependent hydroxylation of Pro402 and/or Pro564 of HIF-1α. To analyze whether SSAT1 is involved in the PHD2-VHL pathway, cells were cotransfected with expression vectors encoding either wild-type FLAG-HIF-1α or the hydroxylation-resistant P402A mutant of HIF-1α and with PHD2, SSAT1, SSAT2, or empty vector. SSAT2, PHD2, or SSAT1 reduced or eliminated coexpression of wild-type FLAG-HIF-1α (Fig. 3A, third through fifth lanes). In contrast, SSAT1 (but not SSAT2 or PHD2) reduced the levels of FLAG-HIF-1α(P402A/P564A) (Fig. 3A, seventh through ninth lanes) despite the much lower levels of V5-SSAT1 compared with V5-SSAT2. Reporter gene transcriptional assays showed that whereas both SSAT1 and PHD2 inhibited p2.1 transcription mediated by wild-type FLAG-HIF-1α, only SSAT1 inhibited p2.1 transcription mediated by FLAG-HIF-1α(P402A/P564A) (Fig. 3B). Thus, SSAT1 shares with SSAT2 the ability to inhibit HIF-1α protein expression and HIF-1-dependent transcription. However, in marked contrast to SSAT2, SSAT1 mediates this effect by a mechanism that is independent of prolyl hydroxylation.

Arg101 is conserved in all metazoan members of the SSAT family and is required for binding of acetyl-CoA and acetylation of spermine and spermidine as determined by both mutagenesis and structural analysis (35–37). We therefore introduced the conservative missense mutation R101K into human SSAT1. The mutant SSAT1(R101K) protein was markedly impaired in its ability to promote HIF-1α degradation (Fig. 4A, upper panel) and to inhibit HIF-1-dependent transcription (Fig. 4B), despite the fact that it
was expressed at higher levels than the wild-type protein (Fig. 4A, middle panel). These results are consistent with the hypothesis that the catalytic activity of SSAT1 as an N-acetyltransferase is required for it to promote HIF-1α degradation.

We recently described an alternative pathway for the ubiquitination and degradation of HIF-1α that does not involve binding of VHL to prolyl-hydroxylated HIF-1α, but instead involves binding of HIF-1α to RACK1, which recruits the Elongin C ubiquitin-ligase complex (23). Compared with expression of shSNC, that of either shSSAT1 or shRNA directed against RACK1 (shRACK1) resulted in FLAG-HIF-1α levels that were increased to a similar degree (Fig. 5, second through fourth lanes). Expression of V5-SSAT1 promoted the degradation of FLAG-HIF-1α, and this effect was counteracted by expression of shSSAT1, as expected (Fig. 5, sixth lane). However, shRACK1 also blocked V5-SSAT1-mediated degradation of HIF-1α (Fig. 5, seventh lane), indicating that the effect of SSAT1 on HIF-1α is RACK1-dependent.

To determine whether SSAT1 and RACK1 interact, purified GST or GST-RACK1 was incubated with lysates prepared from cells transfected with empty vector, wild-type (WT) SSAT1 vector, or mutant SSAT1 (R101K) vector and then captured on glutathione-Sepharose beads. Pulldown products and WCL were analyzed by SDS-PAGE and immunoblot assay using antibody against HSP90, RACK1, GST, V5, or β-actin. After 24 h, cells were treated with vehicle (Veh) or 0.5 μM 17-AAG for 16 h. WCL were prepared and analyzed by immunoblot assay with antibody recognizing FLAG or β-actin.

FIGURE 7. SSAT1 increases HIF-1α/RACK1 interaction and is required for 17-AAG-induced HIF-1α degradation. A, GST-HIF-1α (1–329) was incubated for 2 h at 4 °C with WCL from cells transfected with empty vector, wild-type (WT) SSAT1 vector, or mutant SSAT1 (R101K) vector and then captured on glutathione-Sepharose beads. Pulldown products and WCL were analyzed by SDS-PAGE and immunoblot assay using antibody against HSP90, RACK1, GST, V5, or β-actin. B, cells were cotransfected with FLAG-HIF-1α and vector encoding shSNC (SNC) or shSSAT1 (shS1). After 24 h, cells were treated with vehicle (Veh) or 0.5 μM 17-AAG for 16 h. WCL were prepared and analyzed by immunoblot assay with antibody recognizing FLAG or β-actin.
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Oxygen Dependent Pathway

Hydroxylation

Oxygen Independent Pathway

HIF-1α Ubiquitination

26S Proteasome-Dependent
HIF-1α Degradation

FIGURE 9. Involvement of SSAT1 and SSAT2 in alternative pathways for ubiquitination and degradation of HIF-1α. Left, SSAT2 is required for O2- and PHD2/VHL-dependent ubiquitination of HIF-1α. Right, SSAT1 is required for O2-independent and RACK1-dependent ubiquitination of HIF-1α in response to treatment with the HSP90 inhibitor 17-AAG. In both cases, ubiquitination is mediated by recruitment of the Elongin C ubiquitin-ligase complex, which also includes Elongin B, RBX1, cullin-2, and an E2 ubiquitin carrier protein (B/R/C/E2). bHLH, basic helix-loop-helix domain; ODD, oxygen-dependent degradation domain; TAD, transactivation domain.

expression, also affects the binding of SSAT1 to HIF-1α. GST-HIF-1α-(1–329) was incubated with in vitro translated wild-type SSAT1 or SSAT1(R101K) and then pulled down with glutathione-Sepharose beads. Remarkably, SSAT1(R101K) exhibited increased binding to GST-HIF-1α-(1–329) compared with wild-type SSAT1 (Fig. 6D). These results indicate that the inability of SSAT1(R101K) to negatively regulate HIF-1α is not due to the inability of the mutant protein to interact with HIF-1α.

RACK1 competes with HSP90 for binding to the PAS A-subdomain of HIF-1α (23). To determine whether SSAT1 affects this competition, GST-HIF-1α-(1–329) was incubated with lysates from cells transfected with empty vector or vector encoding wild-type SSAT1 or SSAT1(R101K). Expression of wild-type SSAT1 increased the binding of RACK1 and decreased the binding of HSP90 to GST-HIF-1α-(1–329) without changing the levels of RACK1 or HSP90 protein in the cell lysate (Fig. 7A). SSAT1(R101K) also induced the pulldown of RACK1, suggesting that SSAT1(R101K) can interact with HIF-1α (1–329) and RACK1. However, RACK1 recruited by SSAT1(R101K) did not displace HSP90 from HIF-1α (1–329).

HSP90 inhibitors such as 17-AAG have anticancer effects and induce the degradation of HIF-1α by a mechanism that is independent of PHD2 and VHL activity (21, 22) but dependent on RACK1 (23). To determine whether 17-AAG-induced degradation of HIF-1α is dependent on SSAT1, cells were transfected with vector encoding shSNC or shSSAT1 and treated with vehicle or 17-AAG. Compared with cells expressing shSNC, HIF-1α levels were increased in cells expressing shSSAT1, and HIF-1α levels were reduced by 17-AAG in cells expressing shSNC, but not in cells expressing shSSAT1 (Fig. 7B), similar to results observed with shRACK1 (23). Thus, both SSAT1 and RACK1 are required for the degradation of HIF-1α induced by 17-AAG.

We hypothesized that the increased interaction of RACK1 and HIF-1α in SSAT1-overexpressing cells should lead to increased ubiquitination of HIF-1α. To test this hypothesis, 293 cells were cotransfected with expression vector encoding FLAG-HIF-1α and empty vector or vector encoding wild-type SSAT1 or SSAT1(R101K) and treated with MG132 to block the proteasomal degradation of ubiquitinated HIF-1α. Lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were subjected to immunoblot assay using anti-ubiquitin antibody. Wild-type SSAT1 increased the ubiquitination of HIF-1α, whereas SSAT1(R101K) had little effect (Fig. 8A), despite the fact that SSAT1(R101K) was expressed at
higher levels than wild-type SSAT1 (Fig. 8B). Taken together with the data presented in the preceding figures, these results indicate that wild-type SSAT1 promotes the RACK1-dependent ubiquitination and degradation of HIF-1α.

**DISCUSSION**

In this study, we have demonstrated by gain-of-function and loss-of-function approaches that SSAT1 plays an essential role in the RACK1-mediated ubiquitination and degradation of HIF-1α that is induced by treatment of cells with the HSP90 inhibitor 17-AAG. SSAT1 interacts with both RACK1 and HIF-1α, thereby stabilizing the interaction between these two proteins, which is necessary for the ubiquitination of HIF-1α by the Elongin C ubiquitin-ligase complex that is recruited to HIF-1α by RACK1 (23). In this and previous studies (29, 30), we have now identified three HIF-1α-binding proteins whose multivalent interactions are required for the efficient ubiquitination and degradation of HIF-1α: OS-9, SSAT2, and SSAT1 stabilize PHD2/HIF-1α, VHL/Elongin C, and HIF-1α/RACK1 interaction, respectively (Fig. 9). Whereas OS-9 and SSAT2 are required for O2/PHD2/VHL-dependent ubiquitination, SSAT1 is required for O2-independent and RACK1-dependent ubiquitination of HIF-1α in 17-AAG-treated cells. These studies have revealed the existence of multiprotein complexes that are devoted to the cooperative regulation of HIF-1α stability in response to changes in oxygen concentration and perhaps other physiological stimuli.

It is remarkable that the human SSAT1 and SSAT2 proteins, which share 46% amino acid sequence identity and are clearly homologous, both interact with HIF-1α and both promote the ubiquitination and degradation of HIF-1α, yet they do so by completely different and complementary molecular mechanisms. In particular, SSAT2 binds to the PAS A-subdomain of HIF-1α (residues 81–200) and promotes O2-dependent regulation, whereas SSAT1 binds to the PAS B-subdomain (residues 201–329) and promotes O2-independent regulation of HIF-1α.

It remains to be determined whether the acetyltransferase activity of either SSAT1 or SSAT2 is required for their regulation of HIF-1α. The mutation of a critical arginine residue (R101K) in the acetyl-CoA-binding site of SSAT1 that disrupts its acetyltransferase activity also abrogated its ability to induce HIF-1α degradation. However, despite devoting considerable experimental effort to test the hypothesis that SSAT1 acetylates either HIF-1α or RACK1, we have not yet generated compelling data in support of this hypothesis. Based on these negative results, it is possible that, in addition to affecting acetyl-CoA binding, the R101K mutation has other unrelated effects on the conformation and/or function of SSAT1 that are more relevant to its ability to regulate HIF-1α.

HIF-1 regulates genes that play important roles in several critical aspects of cancer biology, including angiogenesis, glucose metabolism, invasion, metastasis, and resistance to therapy (38–42), and efforts to develop anticancer agents that target HIF-1 are actively under way (43). A promising therapeutic approach that results in inhibition of HIF-1α expression is the use of HSP90 inhibitors such as 17-AAG. HSP90 competes with RACK1 for binding to the PAS A-domain of HIF-1α. HSP90 inhibition leads to increased RACK1 binding and recruitment of the Elongin C ubiquitin-ligase complex that mediates ubiquitination and proteasomal degradation of HIF-1α (23). We have now demonstrated that the efficient interaction of RACK1 with HIF-1α requires SSAT1 and that 17-AAG-mediated degradation of HIF-1α is impaired when SSAT1 levels are reduced by RNA interference. In this and our previous study (23), we have delineated a more detailed mechanism of action by which 17-AAG induces HIF-1α degradation than for any other HSP90 client protein.

Treatment of colon cancer cells with an inducer of SSAT1 expression has been shown recently to increase sensitivity to chemotherapeutic agents (44). HIF-1α-deficient cells manifest increased sensitivity to chemotherapeutic agents (45). HIF-1 has been shown to regulate transcription of the ABCB1 and ABCG2 genes, which encode the MDR1 (multidrug resistance protein-1) and BCRP (breast cancer resistance protein) multidrug transporters, respectively (46, 47). Reduced HIF-1α levels may therefore contribute to the improved chemosensitivity associated with increased SSAT1 expression. Inducers of SSAT1 expression may have synergistic effects with HSP90 inhibitors in reducing HIF-1α levels and thereby increasing sensitivity to conventional chemotherapy.

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