Hsp90 Regulates a von Hippel Lindau-independent Hypoxia-inducible Factor-1α-degradative Pathway*

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Jennifer S. Isaacs, Yun-Jin Jung, Edward G. Minnaugh, Alfredo Martinez, Frank Cuttitta, and Leonard M. Neckers†
From the Cell and Cancer Biology Branch, Center for Cancer Research, NCI, National Institutes of Health, Rockville, Maryland 20850

HIF-1α is a normally labile proangiogenic transcription factor that is stabilized and activated in hypoxia. Although the von Hippel Lindau (VHL) gene product, the ubiquitin ligase responsible for regulating HIF-1α protein levels, efficiently targets HIF-1α for rapid proteasome-dependent degradation under normoxia, HIF-1α is resistant to the destabilizing effects of VHL under hypoxia. HIF-1α also associates with the molecular chaperone Hsp90. To examine the role of Hsp90 in HIF-1α function, we used renal carcinoma cell (RCC) lines that lack functional VHL and express stable HIF-1α protein under normoxia. Geldanamycin (GA), an Hsp90 antagonist, promoted efficient ubiquitination and proteasome-mediated degradation of HIF-1α in both normoxia and hypoxia. Furthermore, HIF-1α point mutations that block VHL association did not protect HIF-1α from GA-induced destabilization. Hsp90 antagonists also inhibited HIF-1α transcriptional activity and dramatically reduced both hypoxia-induced accumulation of VEGF mRNA and hypoxia-dependent angiogenic activity. These findings demonstrate that disruption of Hsp90 function 1) promotes HIF-1α degradation via a novel, oxygen-independent E3 ubiquitin ligase and 2) diminishes HIF-1α transcriptional activity. Existence of an Hsp90-dependent pathway for elimination of HIF-1α predicts that Hsp90 antagonists may be hypoxic cell sensitizers and possess antiangiogenic activity in vivo, thus extending the utility of these drugs as therapeutic anticancer agents.

Hypoxia-inducible factor-1α (HIF-1α) is a component of a transcriptional complex that is extremely labile under normoxia but is stabilized and activated under hypoxia (1). Because HIF-1α regulates a variety of processes such as angiogenesis and glucose metabolism, it is acknowledged to be a critically important tumor cell survival factor that is required for tumorigenesis in many cancer models and is expressed in a majority of metastases and late stage tumors. The rapid degradation of HIF-1α in normoxic cells is mediated by the tumor suppressor VHL, which, together with a multimeric protein complex, serves as its E3 ubiquitin protein ligase (2–5). Under hypoxic conditions, the stabilization and activation of HIF-1α is due to an inability of VHL to associate with and ubiquitinate HIF-1α. It was recently shown that hypoxic conditions impair the ability of a class of enzymes termed prolyl hydroxylases to modify two separate consensus proline motifs present on HIF-1α (6–10). These modifications are required for VHL to associate with and ubiquitinate HIF-1α, thereby targeting the protein for proteasome-dependent degradation.

HIF-1α is constitutively stabilized in normoxic tumors and in cell lines that are VHL null or that express a nonfunctional mutant form of VHL. This occurs in over 50% of sporadic RCCs and clear cell RCCs (11), the most common malignant neoplasm of the kidney, and one of the few human tumors known to depend upon VHL inactivation. The importance of VHL function is further demonstrated in VHL disease, a human cancer syndrome caused by hereditary loss of VHL gene function, resulting in constitutive up-regulation of hypoxically induced genes, and characterized by highly vascular tumors of the central nervous system, in addition to RCCs (12). The crucial role of HIF-1α in tumor progression is underscored by its expression in a significant proportion of breast, colon, prostate, and a variety of other cancers (13–16). The ability of HIF-1α to promote both tumor cell survival and angiogenesis (17–20) strongly suggests that HIF-1α overexpression is important for tumor vascularization and metabolic adaptation to hypoxia (21–23), both essential events for malignant tumor progression. This hypothesis is strengthened by the observations that HIF-1α expression correlates with tumor grade and vascularity (24), and that VHL-inactivated tumors are highly vascular and overproduce angiogenic factors such as VEGF (25–27). VEGF, one of the most potent angiogenic cytokines, is transcriptionally regulated in large part by HIF-1α (28), suggesting that the ability to down-regulate HIF-1α expression would have a positive impact on cancer control.

Geldanamycin (GA) is a naturally occurring anasamycin antibiotic that possesses antitumor properties (29, 30) by virtue of its ability to associate and interfere with Hsp90 function (31). Hsp90 associates with client proteins in a nucleotide-dependent manner, and GA interferes with this association by occupying the nucleotide binding site of Hsp90 (32–34). Hsp90 substrates are numerous and include multiple transcription factors (aryl hydrocarbon receptor, glucocorticoid receptor, Myo D, p53) (35–38) and an array of signaling kinases (Akt, ErbB2,
Raf-1, v-Src) (39–42). Hsp90 plays an essential role in facilitating the proper conformation, localization, and function of these client proteins (43–47), and GA-mediated Hsp90 dissociation from client proteins results in their ubiquitination and subsequent degradation by the proteasome (48–50). Although it is known that HIF-1α associates with Hsp90 (51), a role for this association has remained elusive. Here, we demonstrate that disruption of HIF-1α/Hsp90 association promotes the ubiquitination and proteasome-mediated degradation of HIF-1α in a manner that is both oxygen- and VHL-independent, thereby delineating a novel pathway that regulates HIF-1α protein stability and function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—RCC lines: Caki-1 cells were obtained from ATCC; UMRC2 were provided by Dr. M. I. Lerman (NCI, National Institutes of Health, Frederick, MD); UMRC6 were obtained from Dr. B. Zbar (NCI, Frederick, MD); and 786-O were provided by Dr. R. Klausner (NCI, Bethesda, MD). The designations herein of C2 and C6 refer to UMRC2 and UMRC6. All cell lines were cultured in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum plus 1× nonessential amino acids and penicillin/streptomycin. Cells were treated, as indicated with the following agents: 50 μM N-acetyl-Leu-Val-Lys-Leu-Pro-Arg-Arg-NH2 (ALLnL, Sigma), 2 μM 17-allylamino-17-demethoxygeldanamycin (17-AAG) (both obtained from NCI), 40 μg/ml cycloheximide (CHX, Sigma), 5 μM PS-341 (Millennium Pharmaceuticals), and 100 μM cobalt chloride (Sigma).

**Hypoxic Treatment**—Cells were placed in a 37°C pre-equilibrated Eton II sealed chamber (Sheldon Labs), and premixed gas (94% N2, 5% CO2, 1% O2), was infused to create a hypoxic environment. The oxygen content inside the chamber was constantly monitored by a previously calibrated oxygen sensor (Animas Corp., Frazer, PA). Cells were manipulated and lysed inside the chamber, and all buffers were pre-equilibrated to 1% O2.

**Subcellular Fractionation**—Cells were washed with phosphate-buffered saline and overlaid for 10 min with low salt buffer (10 mM HEPES, 10 mM KC1, 0.1 mM EDTA) containing protease inhibitors. The cells were then scraped into microcentrifuge tubes, and 10% Nonidet P-40 was added (final concentration 0.5%). The tubes were vigorously vortexed for 10 s and spun at 3000 rpm for 1 min, and nuclear pellets were washed with low salt buffer prior to lysis in high salt lysis buffer (20 mM HEPES, 300 mM NaCl, 1 mM EDTA).

**DNA Manipulation and Transfection**—A CMV-driven HA-tagged HIF-1α construct was originally obtained from Dr. D. Livingston (Dana-Farber Cancer Institute, Boston, MA). Using primers containing sites for BamHI (5') and NorI (3'), this insert was PCR-amplified, subcloned into PCDNA3.1 (Invitrogen), and confirmed by sequencing. This latter construct was used for all wt HIF-1α transfections and as a template for subsequent mutations. To make site-specific mutations, complementaries primers containing desired point mutations were constructed, and PCR amplification was performed in accordance with Promega’s XL site-directed mutagenesis kit. For transfection, cells were seeded at 60% confluency 24 h prior to transfection, and 3 μg of plasmid DNA was used per 10-cm dish. Transfections were carried out using FuGENE (Roche Molecular Biochemicals), according to the manufacturer’s specifications.

**Immunoblot Analysis**—Following cell lysis and clarification by centrifugation, equal amounts of protein were loaded onto 7.5% PAGE gels (Bio-Rad). The following antibodies were used: murine HIF-1α, 1:300 (Transduction labs); murine HA, 1:2000 (Covance); rat Hsp90, 1:2000 (Stressgen); murine topoisomerase II, 1:5000 (Sigma); and rabbit polyclonal ubiquitin, 1:1000 (Sigma). For protein visualization, horseradish peroxidase-linked secondary antibodies were used with the ECL protein detection system (Pierce). For HA immunoprecipitations, lysates were incubated with HA-conjugated Sepharose beads (Covance) and for HIF-1α immunoprecipitations, lysates were incubated with HIF-1α antibody, followed by addition of protein G-Sepharose beads (Invitrogen). To detect HIF-1α association with a low molecular weight agent (0.5 M P-40) lysis buffer was used and 20 mM Na2MoO4 was added to stabilize protein interactions. Beads were washed four times with lysis buffer, boiled in Laemmli buffer, and processed as described.

**Pulse-Chase Analysis**—Logarithmically growing UMRC2 cells were starved 30 min in methionine and cysteine-free medium (Invitrogen) and 150 μCi/ml methionine/cysteine (Tran3S-label, ICN) was added for 1 h. After the labeling period, cells were washed with nonradioactive complete medium and incubated in this medium for the indicated times. The cells were then lysed and precleared with protein G beads, and HIF-1α was immunoprecipitated from 1 mg of soluble lysed protein overnight. For GA-treated samples, 2 μM GA was added to the starve medium, retained throughout the labeling period, and included in the chase medium by subjecting media preparations containing known concentrations of GA to filtration and measuring the drug remaining in the retentate by high-performance liquid chromatography, as previously described (52). It was determined that the amount of GA remaining after two successive filtration spins was negligible when the starting concentration did not exceed 0.5 μM. The final filter retentate was concentrated 3-fold for use in the angiogenesis assay.

For the angiogenesis assay, a modified aortic ring method (80) was used. Briefly, aortic rings were prepared from 13-day-old chicken embryos. Each aortic ring was placed in the center of a well and overlaid with Matrigel (BD Biosciences, Bedford, MA) and growth factor-free human endothelial-SFM basal growth medium (Invitrogen) to which a 1:1 volume of cellular serum-free concentrated medium was added. For a positive control, 10 nM bFGF was added, while basal medium alone was used as a negative control. In addition, filtered conditioned medium from GA-treated cells was added to the positive control to ensure that potential trace amounts of GA were not inhibitory in this assay. The aortic rings were incubated at 37 °C in 5% CO2 for 36 h in the presence of conditioned medium, and microvessels sprouting from the aortic rings were photographed with an inverted microscope. All conditions were performed in duplicate. In each case, the degree of angiogenesis was normalized to the area of the corresponding ring. For relative comparison, the degree of angiogenesis for each condition was normalized to the negative control.

**RESULTS**

**GA Promotes the Down-regulation of HIF-1α under Normoxia and Hypoxia**—GA mediates the dissociation of Hsp90 from client proteins and promotes their rapid degradation by the proteasome. Several reports have demonstrated an interaction between HIF-1α and Hsp90 (51, 53), although a definitive biological role for this interaction has remained elusive. We therefore investigated whether Hsp90 might regulate...
HIF-1α protein stability in the absence of VHL. To address this question, we used the RCC lines C2 and C6 (3, 54) that lack a functional VHL protein and therefore express an inherently stable HIF-1α protein under normoxia. As shown in Fig. 1A (upper panel), HIF-1α protein was highly expressed under normoxia in untreated cells but was down-regulated in a dose-dependent manner in response to GA treatment. It appeared that HIF-1α from C2 cells was less sensitive to the effects of GA when compared with the protein from C6 cells, prompting us to extend the duration of drug treatment. As shown in Fig. 1A (middle panel), this prolonged treatment enhanced the sensitivity of HIF-1α to GA, so that the effective GA concentration for both cell lines occurred within the 0.1–0.5 μM range. The lowest panel of Fig. 1A shows that GA promoted the down-regulation of HIF-1α in a time-dependent manner, with the protein in both cell lines diminishing over 6–8 h. To verify the ability of GA to down-regulate HIF-1α levels, 786-O cells deficient for both HIF-1α and VHL (3) were transfected with wild-type HIF-1α and protein levels were assessed. As shown in Fig. 1B, GA treatment resulted in a rapid decrease in HIF-1α protein levels, with over 50% of the protein being eliminated within 1 h of treatment.

Under hypoxic conditions, the normally labile HIF-1α protein is stabilized in VHL-competent cells. It was therefore of interest to determine whether hypoxia could antagonize the effect of GA in cells expressing or lacking functional VHL. Caki-1 is an RCC line that contains wild-type VHL, and in these cells HIF-1α is induced by hypoxia (3). Caki-1 cells were treated with GA while under hypoxia, subsequent to hypoxia-induced stabilization of HIF-1α. Similarly, VHL-deficient C6 cells were treated with GA under normoxia or hypoxia. As shown in Fig. 1C, treatment of cells with GA or its similarly acting, clinically administered analog 17-AAG (55–57), significantly decreased HIF-1α protein levels, regardless of oxygen tension.

To exclude the possibility that the effects of GA on HIF-1α were nonspecific, we examined the association of HIF-1α with Hsp90 in the presence or absence of drug. In accordance with other reports (40, 58), the GA-mediated down-regulation of HIF-1α would be expected to be preceded by dissociation of the HIF-1α-Hsp90 chaperone complex. As shown in Fig. 1D, treatment of either C2 or Caki-1 cells with GA led to a marked decrease in the amount of HIF-1α associated with Hsp90, and GA was equally capable of displacing Hsp90 from HIF-1α independent of oxygen tension. Importantly, oxygen level had no effect on the ability of HIF-1α to associate with Hsp90.
an increase of total HIF-1α levels in the nuclear soluble fraction, demonstrating that HIF-1α is degraded by the proteasome, even in the absence of VHL. No HIF-1α protein was detected in the cytosolic soluble fraction (data not shown).

Compared with GA treatment, the combination treatment stabilized nuclear soluble HIF-1α levels, but to a degree still significantly less than that observed with ALLnL alone. Reports have indicated that treatment of cells with Hsp90 antagonists in combination with proteasome inhibitors results in detergent-insoluble client proteins (58, 59). Therefore, we assessed HIF-1α levels in nuclear and cytosolic detergent-insoluble fractions. As shown in Fig. 3A (middle and right panels), in the absence of treatment, minimal HIF-1α was detected in either insoluble fraction, and treatment with ALLnL alone resulted in only a small increase in insoluble HIF-1α. Although no HIF-1α protein was detected in the insoluble fractions subsequent to GA treatment alone, the combination of GA and ALLnL increased HIF-1α protein in both nuclear and cytosolic detergent-insoluble fractions. In sum, these data show that GA-mediated degradation of HIF-1α occurred by a proteasome-dependent pathway and shares characteristics with the degradation of other Hsp90 client proteins.

Previous reports have demonstrated that GA-mediated proteasomal degradation of Hsp90 client proteins is preceded by their ubiquitination (48, 58, 60, 61). Therefore, we tested whether HIF-1α was similarly ubiquitinated in GA-treated cells prior to its degradation. This was of special interest, because VHL, which serves as the primary ubiquitin ligase (3–5), is nonfunctional in the RCC lines studied. As shown in Fig. 3B, although no HIF-1α-ubiquitin conjugates were observed in untreated cells, these species were easily detected in HIF-1α immunoprecipitates after 3 h of GA treatment, with maximal ubiquitination occurring by 6 h.

To enhance visualization of HIF-1α-ubiquitin conjugates, we treated cells for 0.5 h with the specific proteasome inhibitor PS-341 (62), either alone or in combination with GA. The brief exposure to PS-341 was used to avoid formation of detergent-insoluble HIF-1α that occurs after prolonged treatment with GA in the presence of proteasome inhibitors (see Fig. 3A). As shown in Fig. 3B, the combination treatment dramatically increased HIF-1α-ubiquitin conjugates (detectable by 1 h, data not shown). Although PS-341 alone increased the amount of ubiquitinated HIF-1α species, this increase was significantly less (~8-fold) when compared with that elicited by the combination of GA and PS-341. These data suggest that the HIF-1α ubiquitination/degradation process set in motion by GA is relatively rapid and rather efficient. Interestingly, although the molecular mass of HIF-1α is ~116 kDa, the majority of the ubiquitinated protein in HIF-1α immune precipitates migrated with an apparent molecular mass of ~180 kDa, suggesting that most of the ubiquitinated HIF-1α protein was polyubiquitinated.

To confirm that this high molecular weight species represented ubiquitinated HIF-1α, 786-O cells were transfected with an HA-tagged HIF-1α construct and the experiment described in Fig. 3B was repeated. HIF-1α was immunoprecipitated, and the protein was visualized with an anti-HA antibody, as shown...
in Fig. 3C. The time of exposure to GA was shortened because of the ability of GA to rapidly degrade HIF-1α protein in this cell line (see Fig. 1B). There was virtually no detectable ubiquitinated HIF-1α in control or GA-treated cells and only a slight increase in ubiquitinated species after treatment with PS-341 alone. However, similar to the data in Fig. 3B, a significant increase in ubiquitinated HIF-1α species was evident in cells treated with both GA and PS-341, thus confirming that the HIF-1α protein identified with a ubiquitin antibody in Fig. 3C represented ubiquitinated HIF-1α.

Recently, HIF-1α was shown to undergo proline hydroxylation at two sites (Pro-402 and Pro-564), and these modifications are required for VHL association and thus VHL-mediated degradation of HIF-1α (6, 8–10). To confirm that GA-induced degradation of HIF-1α was independent of proline hydroxylation, we mutated these two residues, either independently or together, and assessed the GA sensitivity of the mutated proteins in VHL-deficient 786-O cells. As shown in Fig. 3D, the mutant proteins were as sensitive to GA as was wild-type HIF-1α. To determine whether these mutant proteins could be ubiquitinated, we transfected 786-O cells with wild-type or proline-mutated HIF-1α constructs, treated the cells with GA, and nuclear lysates were examined for HIF-1α expression. E, cells were transfected as in D, treated with GA, PS-341, or a combination, HIF-1α was immunoprecipitated, and resultant blots were probed with an anti-ubiquitin antibody. The Control lane represents untransfected cells subjected to the combination treatment.

GA Interferes with HIF-1α Transcriptional Activity—The increased transcriptional activity of HIF-1α that occurs during hypoxia is associated with a concomitant increase in protein stability but also depends upon hypoxic inhibition of an asparagine hydroxylation modification that facilitates the associa-
tion of HIF-1α with cofactors such as p300 (63, 64). Once activated, HIF-1α transactivates its target genes by associating with their hypoxia-responsive elements (HREs) (65, 66). One of the numerous transcriptional targets of HIF-1α is VEGF (28), a potent proangiogenic cytokine. Because GA down-regulated HIF-1α protein levels, we determined whether GA could also interfere with HIF-1α transcriptional activity by treating both normoxic RCC lines and hypoxic VHL-competent Caki-1 cells with GA. As shown in Fig. 4A (upper panel), GA treatment of the RCC lines resulted in a modest decline (33–36%) in VEGF165 mRNA by 6 h, with more than a 70% reduction after 16 h (see table in Fig. 4). Similarly, in Caki-1 cells, a 6-h treatment with GA following a 6-h hypoxic pretreatment resulted in a 57% decline in VEGF mRNA, which progressed to a 71% decline after 16 h.

It remained unclear whether GA affected VEGF transcription directly via inhibition of HIF-1α transcription or whether VEGF mRNA expression was affected by other means (67). To address this issue, we performed transient transfection assays using a luciferase expression plasmid under control of HREs from the iNOS promoter (68). As shown in Fig. 4B (left panel), HIF-1α-dependent luciferase activity in VHL-deficient C2 cells was 3.5-fold higher than in C2 cells stably transfected with VHL, thus confirming the HIF-1α dependence of this reporter. In a second experiment, cells were treated with GA 4 h after transfection, and drug treatment was continued for an additional 4 or 6 h. As shown in Fig. 4B (right panel), GA treatment for 4 h resulted in a modest reduction in HIF-1α-dependent luciferase activity, with over a 50% reduction after 6 h.

VEGF is one of the most potent angiogenic cytokines released by hypoxic tumors (69, 70). Because we observed a GA-mediated inhibition of HIF-1α activity and a corresponding decrease in VEGF transcription, we wished to determine whether GA could mitigate hypoxia-induced angiogenesis. To test this possibility, we collected conditioned medium from VHL-competent Caki-1 cells subjected either to normoxia, 12 h of hypoxia, or 12 h of hypoxia in the presence of GA. GA was removed from the conditioned media by size exclusion filtration, and the drug-free filter retentates were then used in a
chick aortic ring angiogenesis assay, as shown in Fig. 5. The top three panels show various controls. Basic FGF (Fig. 5B) was ~2.5-fold more angiogenic than the negative control (Fig. 5A). Medium from Caki-1 cells treated with GA did not inhibit FGF-induced angiogenesis (Fig. 5C), demonstrating that inhibition of angiogenesis by media from GA-treated cells was not due to traces of drug remaining in the medium. Panels D–F depict the angiogenic activity of medium from Caki-1 cells. Medium conditioned by hypoxic Caki-1 cells (Fig. 5E) was ~3-fold more angiogenic when compared with medium from normoxic cells (Fig. 5D). Supporting and extending our previous results, the angiogenic potential of medium conditioned by hypoxic Caki-1 cells treated with GA (Fig. 5F) was comparable to the negative control (Fig. 5, A and D).

**DISCUSSION**

In this study, we have identified the Hsp90 molecular chaperone as a novel VHL- and oxygen-independent regulator of HIF-1α protein stability. This was demonstrated by the ability of the Hsp90 inhibitors GA and 17-AAG to promote the loss of HIF-1α protein from C2 and C6, two VHL-deficient RCC lines containing constitutively elevated HIF-1α levels in normoxia. Transfected HIF-1α was also markedly destabilized by GA in 786-O cells, which lack both VHL and HIF-1α genes. GA and 17-AAG were equally effective at reducing HIF-1α levels in hypoxia and in normoxia and in the presence or absence of endogenous VHL. Although co-precipitation of Hsp90 with HIF-1α was independent of oxygen tension, this interaction was rapidly disrupted by GA, prior to loss of HIF-1α protein. As is the case for other Hsp90 client proteins (40), when Hsp90 was inhibited by GA, both pre-existing and newly synthesized HIF-1α protein pools became unstable, although the rate of HIF-1α synthesis remained essentially unchanged. Importantly, the HIF-1α degradation that was stimulated by the Hsp90 inhibitors GA and 17-AAG remained proteasome-mediated. As has been reported with other Hsp90-soluble client proteins (58, 59), the combination of proteasome inhibition and Hsp90 inhibition resulted in the accumulation of HIF-1α protein in the detergent-insoluble pellet fraction, where under normal circumstances it was undetectable. The fact that the preponderance of HIF-1α was recovered from the nuclear pellet fraction suggests either that GA-induced HIF-1α degradation occurred primarily in the nuclear compartment or that proteasome inhibition interfered with the nuclear export of HIF-1α protein.

GA-mediated dissociation of HIF-1α from Hsp90 markedly enhances HIF-1α ubiquitination in VHL-deficient cells, thereby demonstrating an essential function for Hsp90 in maintaining HIF-1α stability. Although a reduction in steady-state levels of HIF-1α protein required exposure to GA for several hours, a significant amount of HIF-1α ubiquitination can be seen after as little as 1 h of exposure to the drug (data not shown). As a final proof that GA-induced HIF-1α degradation does not proceed via the VHL pathway, we demonstrate that mutation of prolines 402 and 564 in HIF-1α, which render the protein fully resistant to VHL-mediated ubiquitination and degradation (8–10), fails to protect HIF-1α from GA. These data point to a novel, oxygen-independent E3 ubiquitin ligase, distinct from VHL, that is recruited to HIF-1α upon dissociation of the HIF-1α-Hsp90 complex. Although the mdm2 ubiquitin ligase may serve as an E3 for HIF-1α under certain conditions (68), and GA-induced degradation of mutated p53 requires mdm2 (69), this E3 is unlikely to be the ubiquitin ligase that mediates GA-induced HIF-1α degradation, because dominant negative mdm2 failed to protect HIF-1α protein from the destabilizing effects of GA in RCC cells (data not shown). Furthermore, the Hsp90/Hsp70-binding ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein), which may mediate the GA-stimulated degradation of some Hsp90 client proteins (71, 72), also failed to destabilize HIF-1α when the two proteins were co-transfected into either COS7 or 786-O cells (data not shown). We are currently screening several additional candidate E3 enzymes in an effort to identify the oxygen-independent ubiquitin ligase responsible for GA-induced ubiquitination of HIF-1α.

Although the association of HIF-1α with Hsp90 has been previously documented (51, 73), a physiologically important role for Hsp90 in HIF-1α function has remained elusive. We have now demonstrated that Hsp90 plays a pivotal role as a master regulator of HIF-1α protein stability. In contrast to a previous report (53), we found that Hsp90 retains the ability to associate with HIF-1α under both normoxic and hypoxic conditions. It is possible that the use of chemical mimetics of hypoxia by these investigators, instead of reduced oxygen levels, may explain this discrepancy. Indeed, a recent report demonstrating that hypoxic accumulation of HIF-1α protein is antagonized by GA (74) supports the notion that Hsp90 associates with HIF-1α under both normoxic and hypoxic conditions. However, we propose that, rather than preventing its up-regulation, GA promotes the degradation of HIF-1α under hypoxia.

One of the most clinically relevant aspects of this study is the finding that Hsp90 antagonists interfere in a hypoxia-independent manner with HIF-1α transcription, as measured both by reporter assay and by analysis of VEGF mRNA levels. Although two recent reports have suggested that Hsp90 antagonism inhibits expression of VEGF (75, 76), the contribution of the chaperone in these studies is unclear due to the presence of functional VHL in the cells that were used. Although it remains to be determined whether GA-mediated down-regulation of VEGF mRNA is due solely to interference with HIF-1α transcriptional activity, the kinetics of these two events suggest such a relationship.

Because 17-AAG is currently in clinical trial, these results are of particular interest, in that they indicate that this drug may have antiangiogenic activity in cancer patients. This pre-
diction is supported by our data demonstrating that conditioned medium from hypoxic cells exhibits potent angiogenic activity, whereas medium from hypoxic cells exposed to GA does not. Although this inhibitory activity may not be due solely to effects on VEGF synthesis and secretion, to our knowledge this is the first report demonstrating an inhibitory role for GA in hypoxia-induced angiogenesis. The hypoxic state of a tumor correlates with increased malignancy, metastatic potential, poor prognosis, and resistance to radiotherapy and chemotherapy (77, 78). Our findings demonstrate that GA-induced, Hsp90-dependent elimination of HIF-1α occurs under normoxic and hypoxic conditions and is independent of VHL, thus identifying a novel means of treating tumors overexpressing HIF-1α protein.

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Addendum—A study describing similar destabilizing effects of geldanamycin on HIF-1α protein in prostate cell line was published (79) at the time of submission of this report.

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Role of Hsp90 in HIF-1α Stability

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