The Platelet-derived Growth Factor Receptor α Is Destabilized by Geldanamycins in Cancer Cells*

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The heat shock protein HSP90 serves as a chaperone for receptor protein kinases, steroid receptors, and other intracellular signaling molecules. Targeting HSP90 with ansamycin antibiotics disrupts the normal processing of clients of the HSP90 complex. The platelet-derived growth factor receptor α (PDGFRα) is a tyrosine kinase receptor up-regulated and activated in several malignancies. Here we show that the PDGFRα forms a complex with HSP90 and the co-chaperone cdc37 in ovarian, glioblastoma, and lung cancer cells. Treatment of cancer cell lines expressing the PDGFRα with the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) promotes degradation of the receptor. Likewise, phospho-Akt, a downstream target, is degraded after treatment with 17-AAG. In contrast, PDGFRα expression is not affected by 17-AAG in normal human smooth muscle cells or 3T3 fibroblasts. PDGFRα degradation by 17-AAG is inhibited by the proteasome inhibitor MG132. High molecular weight, ubiquitinated forms of the receptor are detected in cells treated with 17-AAG and MG132. Degradation of the receptor is also inhibited by a specific neutralizing antibody to the PDGFRα but not by a neutralizing antibody to PDGF or by imatinib mesylate (Gleevec). Ultimately, PDGFRα-mediated cell proliferation is inhibited by 17-AAG. These results show that 17-AAG promotes PDGFRα degradation selectively in transformed cells. Thus, not only mutated tyrosine kinases but also overexpressed receptors in cancer cells can be targeted by 17-AAG.

The heat shock protein 90 (HSP90)2 is a molecular chaperone that regulates the maturation and intracellular trafficking of several signaling proteins and receptors, including Her2 neu (1–4), mutant EGFR (5), steroid receptors (6–8), Akt (9), raf (10, 11), src (12), mutant p53 (13), and Bcr-Abl (14, 15). HSP90 plays a key role in the process of protein folding, stabilization, and degradation (16). The chaperone interacts with each substrate forming discrete subcomplexes, which contain different sets of co-chaperones. In cancer cells, HSP90 is present entirely in multichaperone complexes with high ATPase activity, being involved in the processing of oncoproteins critical to cancer progression (17). In contrast, in normal tissue, HSP90 is mostly un-complexed and latent, binding less avidly to client proteins (18). Many of the key signaling molecules deregulated in human cancers require HSP90 to stabilize their conformation and permit their function. In this manuscript, we investigate the role of HSP90 in the processing of the platelet derived growth factor receptor α (PDGFRα), a protein implicated in transformation, particularly in human tumors originating in the mesenchyme. Ansamycin antibiotics bind to the ATP pocket of HSP90, altering the conformation of the multichaperone complex (19, 20). Consequently, HSP90-dependent protein folding and maturation are arrested and proteins are targeted for degradation. In cancer cells, HSP90 inhibitors (geldanamycin, radicicol, herbimycin A, and 17-allylamino-17-demethoxygeldanamycin (17-AAG)), promote the degradation of HSP90-dependent oncoproteins, eventually leading to cell cycle arrest and cell death. Interestingly, geldanamycin derivatives accumulate and bind preferentially to HSP90 in tumor cells compared with normal cells, promoting selective cancer cell death (18). These properties make this class of agents suitable for clinical development as anti-cancer therapeutic agents (21).

The PDGFRα is particularly important to the physiologic functions of the mesenchyme, but its deregulation promotes transformation. For instance, the receptor is overexpressed and/or activated in glioblastomas (22), gastrointestinal stromal tumors (23), medulloblastomas (24), and sarcomas (25). The PDGFRα is also expressed in epithelial cancers, such as ovarian tumors derived from Mullerian elements, which are of dual epithelial/mesodermal origin (26–28) and lung cancers (29). PDGFRα activation in cancer occurs as a consequence of gene amplification (22), chromosomal rearrangements, mutations (23), or autocrine/paracrine engagement (30). Two structurally similar receptor tyrosine kinases (PDGFRα and -β) are activated by PDGFs (20, 31). The receptors mediate signals critical to cell growth and survival (32), transformation (33), migration (34), and vascular permeability (35). Given its role as an oncoprotein necessary for transformation, tumor growth, and progression, we hypothesized that a chaperone complex containing HSP90 may need for its stabilization.

We report here that the PDGFRα forms a complex with HSP90 and the co-chaperone cdc37 in cancer cells. Targeting of
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HSP90 by 17-AAG leads to degradation of the receptor in transformed but not in untransformed cells. This is mediated by ubiquitination of the receptor and its subsequent degradation by the proteasome. A consequence of HSP90 induced PDGFRα degradation is inhibition of PDGFRα-governed cancer cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—17-AAG, PDGF-BB, chloroquine, monensin, leupeptin, and MG132 were purchased from Sigma. PD150606 (calpain inhibitor) was from Calbiochem. PDGF-AA was from PeproTech (Rocky Hill, NJ). The neutralizing antibody to PDGFRα, 3G3, a fully humanized antibody with high affinity for PDGFRα, was a generous gift from Dr. Nick Loizos, ImClone Inc. (36). Rituximab, a humanized anti-CD20 antibody, was from Genentech Inc., San Francisco, CA. Neutralizing anti-PDGFRβ antibody was purchased from Upstate Biotechnology. The neutralizing antibodies to PDGF and to PDGFRα and Rituximab were used at 10 μg/ml during an overnight incubation before treatment with 17-AAG or prior to stimulation with PDGF.

Cell Lines—U118 glioblastoma cells, COS and NIH 3T3 cells (ATCC, Manassas, VA) were grown according to instructions provided by ATCC. Human smooth muscle cells (HSMC) were obtained from Clonetics (Baltimore, MD) and grown according to the manufacturer’s directions. C848 is a primary ovarian cancer culture (gift from Cedars Sinai Ovarian Cancer Repository, Dr. R. L. Baldwin), and the immortalized ovarian cell line C272/hTert/E7 was obtained by transducing the catalytic subunit of human telomerase and the papilloma virus subunit E7 into an ovarian tumor derived primary culture, as described previously (37). Primary and immortalized ovarian cells were grown in growth media containing 1:1 MCDB 105 media (Sigma) and 1% fetal calf serum and 1% penicillin/streptomycin. The SH-SY5Y, a neuroblastoma cell line, was a gift from Dr. Carol Thiele of the National Cancer Institute, National Institutes of Health (38). The Ty5 PDGFRα expressing epithelial cell line established from large cell lung carcinoma, a gift from Dr. George Sledge of Indiana University, was grown in RPMI. R-ras-transformed 3T3 fibroblasts (39) were a gift from Dr. Lawrence Quilliam of Indiana University. All cells were grown in the presence of 10% fetal bovine serum, unless otherwise specified, at 37 °C under 4% CO2 and were harvested during the log growth phase, at 80–90% confluency.

Immunoblotting—Actively growing cells were lysed into RIPA buffer containing leupeptin (1 μg/ml), aprotinin (1 μg/ml), PMSF (400 μM), and Na3VO4 (1 mM). Cell lysates were sonicated and incubated on ice for 30 min. Cellular debris was removed by centrifugation for 15 min. Protein concentration was measured by the Bradford method. Equal amounts of protein from the supernatants (usually 100 μg per sample) were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and treated with a primary antibody: anti-phospho Ser473Akt rabbit antibody (Cell Signaling, 1:1000 dilution), anti-Akt antibody (Cell Signaling, 1:1000 dilution), anti-PDGFRα antibody (Cell Signaling, 1:1000), anti-PDGFRβ antibody (Santa Cruz Biotechnology, 1:250), anti-ubiquitin antibody (Santa Cruz Biotechnology, 1:3000), anti-HSP90 antibody (StressGen 1:500 dilution), anti-cdc37 antibody (Santa Cruz Biotechnology, 1:1000 dilution), anti-PI 3-kinase, p85 antibody (Upstate, 1:5,000 dilution), anti-phospho-PDGFRα antibody (Santa Cruz Biotechnology, 1:500 dilution), anti-β1 integrin antibody (Chemicon, 1:1000 dilution), and anti-GAPDH (Santa Cruz Biotechnology 1:10,000 dilution) during an overnight incubation at 4 °C. After incubation with corresponding horseradish peroxidase-labeled secondary antibodies, enhanced chemiluminescence was used for protein visualization.

Immunoprecipitation—Cells were lysed on ice in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 2.5 mM sodium pyrophosphate, 1% Triton X-100, leupeptin (1 μg/ml), aprotinin (1 μg/ml), and PMSF (400 μM). Alternatively, a second buffer containing 10 mM Hepes (pH 7.4), 150 mM KCl, 10 mM MgCl2, 0.1% Nonidet P-40, 20 mM β-glycerophosphate, 20 mM sodium molybdate, 1 mM sodium orthovanadate, leupeptin (1 μg/ml), aprotinin (1 μg/ml), and PMSF (400 μM) was used to demonstrate the association of proteins with HSP90 (40). After 15 min, lysates were centrifuged at 13,000 rpm for 30 min to pellet cellular debris. 500 μg of protein from the supernatant was incubated overnight at 4 °C with anti-PDGFRα or with anti-cdc37 antibodies, then with 30 μl of a slurry of Protein G Plus-agarose beads for 90 min at 4 ºC. Rabbit or mouse specific IgG (Santa Cruz Biotechnology) were used as a control for immunoprecipitation, depending on the antibody used for immunoprecipitation. Protein-antibody-bead complexes were centrifuged in a wash buffer containing 0.2% Triton and then boiled for 5 min in 1× SDS protein loading dye.

Immunofluorescence—Cells plated on fibronectin-coated glass coverslips (BD Biosciences) were treated with 17-AAG and/or MG132 for 6 h, then fixed with 3.7% paraformaldehyde and permeabilized using Triton X-100 (0.2% in PBS; 10 min). After blocking with 3% goat serum in PBS, cells were incubated with anti-PDGFRα antibody (Santa Cruz Biotechnology, 1:50) for 2 h at room temperature, followed by a 30-min incubation with Alexa Fluor 488 anti-rabbit secondary antibody (1:1000, Molecular Probes, Eugene, OR). A rabbit isotype-specific IgG served as negative control. Nuclei were visualized by DAPI staining (Vectashield, Vector Laboratories, Burlingame, CA). Analysis was performed using a Zeiss LSM510 meta-confocal multiphoton microscope system under UV excitation at 488 nm (for Alexa Fluor 488) and 340 nm (for DAPI).

Separation of Cytosolic and Membrane Cellular Fractions—After treatment, C272/hTert/E7 were collected in a hypotonic buffer containing 10 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 7.4), and 2 mM PMSF. The cell lysate was centrifuged at 4000 × g for 15 min to remove cell debris and nuclei. The supernatant was then centrifuged at 100,000 × g for 60 min to separate the membrane fraction. The final crude membrane pellet was resuspended in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM PMSF.

Transfection—To overexpress the PDGFRα, COS cells in the logarithmic phase of growth were transfected with PDGFRα cloned into the pDualGC vector (Stratagene, La Jolla, CA) using Mirus LT1 (Mirus Biocorporation, Madison, WI). Transfection efficiency is typically 50–60% in these cells, as determined by estimation of green fluorescent protein expression. 24 h after
transfection, cells were harvested and the lysate was used for immunoprecipitation.

siRNA Transfection—For transient transfection with siRNA we used Ty5 cells and HSP90 α- and β-targeted oligomers (41) synthesized by Invitrogen. Control was scrambled siRNA (Dharmacon). Cells were harvested 96 h after transfection. Transfection efficiency in these cells is typically 90%, as estimated by green fluorescent protein expression.

Cell Proliferation—An enzyme-linked immunosorbent assay colorimetric method based on measurement of BrdUrd incorporation into DNA (Roche Diagnostics, Penzberg, Germany) assessed cell proliferation. In brief, after any treatment, cells were incubated with BrdUrd (10 μM) for 3 h and then fixed and denatured. Cells were subsequently treated with a peroxidase labeled anti-BrdUrd antibody for 90 min, and the colorimetric reaction was developed with a tetramethylbenzidine-based substrate. The reaction product was measured in an enzyme-linked immunosorbent assay plate reader (SpectraMax 190, Molecular Devices) at an emission wavelength of 370 nm. All assays were performed in quadruplicate and were repeated twice in independent experiments. Data are presented as means ± S.E.

Statistical Analysis—A two-tailed Student’s t test compared results of cell proliferation assays.

RESULTS

17-AAG Promotes Degradation of the PDGFRα in Human Cancer Cells—We have previously demonstrated that the PDGFRα is over-expressed in ovarian cancer cells (30, 42). To test whether HSP90 is necessary for processing and/or stabilization of the PDGFRα in such cells, we treated cancer cells with 17-AAG and measured by Western blot analysis expression levels of the PDGFRα during 24 h of drug exposure. Treatment with 17-AAG decreased PDGFRα levels in immortalized C272/hTert/E7 and in C848 primary ovarian cancer cells (Fig. 1, A and B). To determine whether this effect was observed in various cancer cell types, the effect of 17-AAG on the receptor was tested in other transformed cells. The PDGFRα levels were reduced by 17-AAG treatment of U118 glioblastoma cancer cells, SH-SY-5Y neuroblastoma cells, and TY5 neuroendocrine lung cancer cells (Fig. 1, C–E). Diminished expression of the receptor was initiated within 1–6 h of treatment depending on the cell line investigated. Thereafter, low expression of the receptor was observed for up to 24 h, without noticeable cytotoxicity. Treatment of cells with 17-AAG also decreased levels of the PDGFRβ in transformed cell lines expressing the β receptor (C848, C272/hTert/E7 and U118). Ty5 and SHSY-5Y cells do not express detectable PDGFRβ. However, the effect of 17-AAG on the PDGFRβ was less pronounced and delayed compared with its effects on the PDGFRα in C272/hTert/E7 and C848 cells.

In parallel with investigation of the effects of 17-AAG on receptor stability, its effects on Akt, a mediator of PDGFRα action (43), were studied. Receptor activation by PDGF leads to recruitment of PI 3-kinase with subsequent phosphorylation of phosphatidylinositol 4,5-biphosphate. PI-3,4,5-triphosphate is formed and binds the pleckstrin homology domain of Akt causing a conformational change that allows Akt activation (phosphorylation) (44). We observed that after treatment with 17-AAG, Ser473 phospho-Akt levels decreased rapidly in cancer cells, and this occurred in parallel with the loss of the PDGFRα (Fig. 1, A–E). Expression of total Akt also diminished after exposure of cancer cells to 17-AAG. The effect of 17-AAG on total Akt lagged behind (by several hours) the diminished expression of the activated (phosphorylated) protein.

Dose-response experiments tested the sensitivity of cells to 17-AAG. Exposure to low doses of 17-AAG (10–100 nM) for 6 h induced loss of PDGFRα and β expression in C272/hTert/E7 ovarian cancer and U118 glioblastoma cells (Fig. 2, A and B). Expression of Ser473 phospho-Akt decreased in parallel with the receptor. Exposure of cells to a higher concentration of 17-AAG was required to induce loss of total rather than activated Akt. In contrast, PI 3-kinase (p85) levels were unaffected by 17-AAG. This suggests that the effects of 17-AAG are target-specific rather than a general effect on activated membrane proteins.

Consistent with experiments utilizing 17-AAG, down-regulation of the HSP90 chaperone complex by siRNA also led to diminished expression of the PDGFRα. In Ty5 cancer cells, decreased receptor levels were noted when HSP90 α, HSP90 β, and HSP90 α + β were knocked down using siRNA, with maximal down-regulation of PDGFRα being observed when both HSP90 α and β were knocked down (Fig. 2C).
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**PDGFRα Expression Is Not Affected by Exposure to 17-AAG in Untransformed Cells**—To test whether the effects of 17-AAG are cancer cell-specific, we examined its effects on untransformed cells. As the normal counterparts of the cancer cell lines utilized here (specifically, human ovarian epithelial or lung epithelial cells) do not express the PDGFRα, we resorted to using HSMC, a human primary culture that expresses the PDGFRα. In HSMC, PDGFRα levels were not significantly affected by treatment with 17-AAG (1 μM) even after 24 h of culture (Fig. 3A). PDGFRβ was not detectable by Western blotting in these cells and therefore the effect of 17-AAG on it was not determined.

Additionally, we examined the effects of 17-AAG on 3T3 fibroblasts. To determine whether 17-AAG affects differentially the PDGFRα in transformed and untransformed cells, we compared the effects of geldanamycin on the PDGFRα in wild type and ras-transformed 3T3 fibroblasts. In untransformed 3T3 cells, PDGFRα and β levels were not affected by exposure to 17-AAG for up to 24 h. In contrast, the PDGFRα level was significantly decreased within 1 h of 17-AAG treatment in ras-transformed fibroblasts (Fig. 3B). These observations suggest that 17-AAG disrupts more efficiently the chaperoning activity of HSP90 in transformed than in untransformed cells.

**PDGFRα Forms a Complex with HSP90 and CDC37 in Cancer Cells**—The effects of 17-AAG on PDGFRα levels suggest that the receptor associates with the HSP90 complex. To test the association between PDGFRα and HSP90, we transfected wild type PDGFRα into COS cells. A cell lysate was prepared, and the PDGFRα was immunoprecipitated with a specific PDGFRα antibody. The Western blot was probed for PDGFRα and for HSP90. Fig. 4A demonstrates interaction between the overexpressed receptor and the chaperone protein, HSP90. We next tested for endogenous interaction between PDGFRα and HSP90 in U118 glioblastoma cells and in untransformed 3T3 cells. Immunoprecipitation with PDGFRα antibody brings down HSP90 in cancer cells but not in untransformed fibroblasts (Fig. 4B).

For other protein kinases, it is known that a co-chaperone, p50 (cdc37), is present in the HSP90 complex (45, 46). Thus, trimers of HSP90, cdc37, and protein kinases exist in cells. To determine whether the PDGFRα associates with such a chaperone complex, we immunoprecipitated cdc37 from lysates of C272/hTert/E7, U118, and Ty5 cancer cells and tested the presence of PDGFRα and HSP90 in the complex by Western blot analysis. The observations in Fig. 4C illustrate that endogenous HSP90 and the PDGFRα co-immunoprecipitate with cdc37.

**17-AAG Induces PDGFRα Degradation by the Proteasome**—In other cell systems and in studies of other receptors, the effects of geldanamycin have been attributed to disruption of the chaperoning activity of HSP90 and subsequent targeting of oncoproteins by the proteasome (9). We tested whether the decreased PDGFRα levels induced by 17-AAG are due to receptor degradation through ubiquitination, which targets the receptor for degradation. First, we tested the effect of the proteasome inhibitor MG132 on PDGFRα expression. We found that loss of PDGFRα expression induced in ovarian cancer cells by a 6-h incubation with 17-AAG was inhibited by pretreatment with MG132 (Fig. 5A). Similarly, treatment with MG132 prevented the degradation of activated Akt induced by 17-AAG, indicating that disruption of the chaperoning activity of HSP90 by 17-AAG, leads to degradation of its clients through the proteasome. Akt was not affected by exposure to 17-AAG for 6 h in these cells; consequently MG132 was without effect on the non-activated kinase. To exclude other potential mechanisms, we pretreated C272/hTert/E7 cells with inhibitors of protein degradation or trafficking prior to geldanamycin treatment. Inhibition of lysosomal proteases (leupeptin) or of calpain (PD150606) did not prevent 17-AAG induced PDGFRα degradation, whereas MG132 rescued PDGFRα from the effects of 17AAG (Fig. 5B). Likewise, treatment with monensin,

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**Figure 3. Effects of 17-AAG in normal HSMC (A) and wild type 3T3 fibroblasts and ras-transformed 3T3 fibroblasts (B).** Cells were treated with 1 μM 17-AAG or vehicle (Me2SO) for 1–24 h. Western blotting with specific antibodies was performed for PDGFRα, PDGFRβ, and GAPDH.
which inhibits intracellular protein trafficking, did not influence the effects of 17-AAG. Interestingly, chloroquine, an inhibitor of receptor recycling, had partial protective effects on the PDGFRα, suggesting that trapping of the receptor in endosomes may prevent its targeting to the proteasome.

To test whether PDGFRα degradation was initiated by ubiquitination, C272/hTert/E7 cells were treated with 17-AAG in the presence or absence of MG132. The combination of MG132 and 17-AAG led to accumulation of high molecular forms of PDGFRα (Fig. 5C, top). By probing a Western blot with an antibody to ubiquitin, we demonstrated that ubiquitinated forms of the receptor are detected in the presence of MG132 and MG132 with 17-AAG (Fig. 5C, middle).

We tested whether disruption of the chaperoning activity of HSP90 by 17-AAG affects the cellular localization and/or expression of the PDGFRα by immunofluorescent staining and confocal microscopy. Treatment of C272/hTert/E7 cells with 17-AAG led to decreased PDGFRα staining at the membrane, while MG132 treatment increased PDGFRα immunoreactivity at the cell membrane and in the cytoplasm (Fig. 6A). Likewise, cell fractionation utilizing ultracentrifugation demonstrated decreased PDGFRα expression in the plasma membrane in cells treated with 17-AAG (Fig. 6B). In contrast, treatment with MG132 prior to 17-AAG led to retained PDGFRα expression in the membrane fraction, consistent with the findings of IF staining. These observations suggest that the receptor, chaperoned by HSP90, is targeted for degradation by 17-AAG and that MG132 blocks this effect.

The Effects of 17-AAG Depend on the Conformation and Activation of PDGFRα—To test whether receptor degradation induced by 17-AAG depends on the activation status of the PDGFRα, we examined the effects of 17-AAG after prestimulation of cells with PDGF or after receptor inactivation. We found that the receptor is degraded after treatment with the geldanamycin derivative in basal conditions (serum starvation) or after PDGF stimulation in C272/hTert/E7 cells (Fig. 7A) or U118 glioblastoma cells (Fig. 7B). To inactivate the receptor we utilized three different modalities: a neutralizing antibody to PDGF-AB, an antagonist antibody to the PDGF receptor, and a receptor tyrosine kinase inhibitor, Gleevec. Both neutralizing antibodies (to PDGF-AB and to PDGFRα) and imatinib prevented receptor activation by PDGF, as demonstrated by Western blotting utilizing a phospho-PDGFRα-specific antibody (Fig. 7C). Neutralization of secreted PDGF by an antibody or inactivation of PDGFRα by the tyrosine kinase inhibitor imatinib prior to treatment with 17-AAG did not
significantly impair the ability of 17-AAG to diminish PDGFRα expression (Fig. 7, A and B). In contrast, neutralization of the PDGFRα by a humanized antagonist antibody (3G3) prevented receptor degradation induced by 17-AAG. This suggests that specific inactivation of the PDGFRα in a manner that may affect its conformation renders the receptor less susceptible to degradation induced by geldanamycin. The effects are observed in both cancer cell lines tested (C272/hTert/E7 and U118) and suggest that the neutralizing receptor antibody has a unique effect on the receptor.

**DISCUSSION**

The present study demonstrates that geldanamycin derivatives induce degradation of the mature PDGFRα. These effects are observed in several cancer cell lines (ovarian, lung, glioblastoma, neuroblastoma), although there are differences in the time and dose dependencies with which transformed cells respond to 17-AAG. In addition, the PDGFRα and PDGFRβ
Both PDGF receptors, α and β, are targeted by 17-AAG in cancer cells. These findings are consistent with studies demonstrating that other trans-membrane receptor tyrosine kinases (i.e. Her2 neu, mutant EGFR) are processed through the HSP90 machinery (5). Interestingly, while the PDGFRα is sensitive to degradation by 17-AAG in cancer cells, the receptor is stable in non-transformed cells (Fig. 3). This may reflect its activation state, the active receptor having a stronger dependence on HSP90 in cancer cells. In contrast, the less active or inactive receptor may interact more transiently with HSP90 in non-transformed cells, as previously demonstrated (18) and as shown by us in wild type 3T3 cells (Fig. 3B) and in HSMC (Fig. 3A). These data are consistent with a previous study which did not demonstrate interaction between PDGFRβ and HSP90 in untransformed Balb/c 3T3 cells. In this study treatment with herbimycin did not affect the mature PDGFRβ but interfered with the processing of the nascent receptor (49). The different effects of 17-AAG on the PDGFRα in ras-transformed and in untransformed 3T3 fibroblasts (Fig. 3B) strengthen the concept that the receptor is heavily dependent on HSP90 in the context of transformation. Last, we cannot exclude that the difference in susceptibility to degradation induced by 17-AAG in untransformed cells may reflect cell type specificity.

Our data suggest that PDGFRα is an HSP90 client. We demonstrate this interaction by overexpressing the wild type receptor in COS cells and showing co-immunoprecipitation with HSP90 (Fig. 4A). The interaction of HSP90 with the endogenous receptor is also demonstrated in U118 cancer cells but did not occur in non-transformed cells. Knowing that in other cell systems, HSP90 forms a hetero-complex with the co-chaperone cdc37 and with receptor tyrosine kinases (46), we assayed whether such complexes containing the PDGFRα are detectable in transformed cells. By immunoprecipitation with anti-cdc37, we show interaction between PDGFRα and cdc37 and between cdc37 and HSP90 in C272/hTert/E7 cells and U118 and Ty5 lung cancer cells. These data demonstrate the presence of complexes containing the PDGFRα, HSP90, and the co-chaperone cdc37 in cancer cells.

Previous studies have not demonstrated interaction between other membrane receptor tyrosine kinases (such as wild type EGFR) and HSP90 (2, 5). In contrast, mutant EGFRs or the variant, EGFRvIII, interact with HSP90 and are sensitive to degradation induced by 17-AAG (5, 46). This report demonstrates for the first time that the wild type, active PDGF receptor α is a substrate for HSP90 in cancer cells. In the cell lines studied the receptor is not mutated. Its activation is due to autocrine stimulation (30) or overexpression, which leads to activation by ligand-independent dimerization (44, 50). Thus we show that not only activating mutations studied by others but also activation by ligand or overexpression render tyrosine kinase oncoprotein substrates for HSP90 stabilization and targets for 17-AAG (shown here).

The effects of 17-AAG on the PDGF-activated PDGFRα are more pronounced than on the inactive receptor in serum-starved conditions (Fig. 7). This may reflect a higher dependence of the activated receptor on HSP90 or may be the consequence of PDGFRα depletion via internalization induced by the interaction with ligand. Also, pretreatment with an antagonist

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**FIGURE 8. 17-AAG inhibits PDGF-induced cell proliferation.** Cell proliferation was assessed by BrdUrd incorporation in C272/hTert/E7 (A) and in U118 cells (B). Cells were serum-starved for 18 h (U118) or 36 h (C272/hTert/E7) prior to treatment with 1 μM 17-AAG. After 6 h of treatment with 17-AAG, cells were stimulated with PDGF-AA and -BB (50 ng/ml). The BrdUrd incorporation assay was carried out 18 h after PDGF stimulation. Columns represent averages of four quadruplicates, and bars are S.E. Statistically significant differences in BrdUrd incorporation were assessed using two-tailed Student’s t test and are denoted with an asterisk.

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...respond differently to 17-AAG, the former receptor being more sensitive to disruption of HSP90 in cancer cells. Blocking the chaperoning activity of HSP90 by geldanamycin leads to receptor degradation through the proteasome, which is inhibited by MG132. The initial step in this process is receptor modification through ubiquitination. We show that the PDGFRα and HSP90 complex in transformed but not in untransformed cells, which explains the capacity of 17-AAG to affect the stability of the receptor. The effects of 17-AAG on the PDGFRα and on other intracellular signaling molecules inhibit cancer cell proliferation in response to PDGF.

This is the first report demonstrating that the PDGFRα is a substrate for HSP90, that this is specific to cancer cells, and that the receptor can be targeted by geldanamycin derivatives. The PDGF receptors are important to the growth and transformation of several malignancies, including glioblastomas, sarcomas, ovarian cancer, and GIST (23, 26, 47). Our group established the role of an autocrine mechanism in activation of the PDGFRα in ovarian tumors (30), and our interest is to develop novel modalities to intercept this growth pathway and to be used as cancer therapy. The present work demonstrates that the PDGFRα and PDGF-induced tumor cell growth are selectively amenable to inhibition by 17-AAG. Whereas many approaches toward therapy seek highly specific reagents, the lesser specificity of 17-AAG, which targets numerous, inappropriately activated targets, offers benefits, one these being that it spares most non-transformed cells.
receptor antibody (3G3), which inhibits PDGF binding to PDGFRα (36) but not receptor inactivation by a tyrosine kinase inhibitor (imatinib mesylate), alters its sensitivity to 17-AAG. The protection from geldanamycin-induced degradation conferred by 3G3 may reflect inhibition of ligand-induced receptor trafficking as a consequence of blocking binding to PDGF and/or a unique conformational change induced by 3G3, rendering the receptor less susceptible to 17-AAG.

Rapid loss of phosphorylated Akt observed in cancer cells after 17-AAG treatment occurred with a time course similar to the decline in PDGFRα levels (Fig. 1). Akt inactivation by geldanamycin has been reported in other cancer cell lines and attributed to activation of the PP1 phosphatase. The activity of the phosphatase is regulated by trans-membrane receptors (i.e. Erb2) and is susceptible to the effects of HSP90 inhibitors on such receptors (51). The effects of 17-AAG on phosphorylated Akt observed here may be partly dependent on PDGFRα degradation upstream of Akt, thereby diminishing a stimulus that induces Akt activation and partly due to effects of AAG on activated Akt, a known HSP90 substrate (1). In contrast, PI 3-kinase (p85) levels are not affected by 17-AAG (Fig. 2), suggesting that geldanamycins target particular proteins that are HSP90 clients and that specific components of signaling pathways are susceptible to inhibition by 17-AAG.

Geldanamycin derivatives target other receptor tyrosine kinases (i.e. Erb2) for ubiquitination and proteasomal degradation (1). We observed that MG132 treatment prevents 17-AAG-induced degradation of the PDGFRα and leads to accumulation of ubiquitinated forms of the receptor (Figs. 5 and 6). This suggests that blocking the chaperoning properties of HSP90 targets the PDGFRα for degradation through the proteasome. This conclusion is supported by the observation that this effect of 17-AAG can be prevented by a proteasome inhibitor but not by other inhibitors of protein degradation (lysosomal or calpain-mediated). As with other receptor tyrosine kinases, regulation of PDGFRα homeostasis is dependent on ubiquitination (44, 52). A consequence of PDGFRα degradation induced by 17-AAG is inhibition of cell proliferation, as 17-AAG prevents PDGF-induced cell proliferation in U118 and C272 cells (Fig. 8). These effects may be a consequence of 17-AAG-induced PDGFRα degradation or may be due to inactivation of other PDGFRα-activated intracellular signaling proteins processed through the chaperone complex.

In summary, the PDGFRα can be targeted by geldanamycin derivatives, and such targeting inhibits cancer cell proliferation. In addition, overexpression of the wild type receptor is sufficient to make it a target for 17-AAG. Therapy targeting the chaperoning activity of HSP90 might be applicable to tumors whose growth is driven by the PDGFRα. Evaluation of HSP90 inhibitors is under way in cancer patients (48).

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