Effect of geldanamycin on androgen receptor function and stability

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Abstract In the ligand-binding inactive state, the steroid receptor heterocomplex contains Hsp90, Hsp70, high-molecular weight immunophilins, and other proteins. Hsp90 acts in association with co-chaperones to maintain the native state of the receptor within the cells. It was reported earlier that Hsp90 might not be as important for the androgen receptor (AR) activity as for the glucocorticoid receptor (GR) and the progesterone receptor (PR) activities. We used the Hsp90 inhibitor geldanamycin (GA) to explore the role of Hsp90 in the function of the AR heterocomplex. GA selectively binds to Hsp90 and inhibits its activity, leading to the loss of steroid receptor activity, and frequently, its degradation. In our study, LNCaP prostate cancer cells were treated with GA for 30 minutes or 24 hours, in the presence of mibolerone, a synthetic androgen. GA reduced the androgen-induced AR protein levels to 15% after 24 hours of treatment. Several androgen up-regulated genes, including immunophilin FKBP51 and prostate specific antigen (PSA), were reduced by GA treatment. In cells treated with GA after transfection with a PSA promoter or an androgen response element-driven reporter gene, AR-mediated transactivation of reporter gene expression was reversibly inhibited by GA. Loss of androgen-binding ability and AR levels was attributed to reduced transcription of AR-regulated gene expression. Degradation rate of ³⁵S-labeled AR was significantly increased by GA in the presence or absence of mibolerone. GA induced the degradation of AR through the proteasomal pathway. AR in cells treated with proteasomal inhibitor lactacystin, was insoluble in Nonidet P-40 (NP40)-based buffer and could not restore the androgen-binding ability. We report here that GA treatment disrupted both hormone-binding activity and receptor protein stability, resulting in a dramatic loss of androgen-induced gene activation. These results show that Hsp90 activity is important for both the chaperone-mediated folding of the AR into a high-affinity ligand-binding conformation and the functional activity of the AR.

INTRODUCTION

A number of transcription factors and protein kinases involved in signal transduction exist in the heterocomplex with the ubiquitous and essential protein chaperone Hsp90 (Pratt and Toft 1997; Buchner 1999). Hsp90 functions as part of a large and dynamic heterocomplex along with a number of cochaperones, including Hip (p48), Hop (p60), p23, Hsp/c70, and the immunophilins, FKBP51, FKBP52, and CyP40 (Milad et al 1995; Pratt et al 1999). Members of the immunophilin family display peptidyl-prolyl isomerase activity, and may play a role in protein folding (Schmid 1993; Pratt 1998). In the steroid receptor heterocomplex, the Hsp90 chaperone system determines proper folding of the hormone-binding domain to pro-

duce a steroid-binding conformation (Pratt 1997). Unliganded androgen receptor (AR) is predominantly cytoplasmic, and exists as a complex with molecular chaperones, including constitutive Hsp70, Hsp90, and HDJ2/HSDJ (Stenoien et al 1999). Thus, the association of receptors with Hsps prevents these receptors from interacting with specific deoxyribonucleic acid (DNA) recognition sequences (or hormone response elements) while they are not yet complexed with the respective steroidal ligands. Ligand binding induces a conformational change in the receptor molecule, which causes it to dissociate from the Hsp complex, and this leads to receptor dimerization, interaction with coactivators, DNA binding, and target gene activation (Evans 1988).

The ansamycins, geldanamycin (GA) and herbimycin-A, are naturally occurring antitumor antibiotics (Whitesell et al 1994). The antitumor effects of GA likely result

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from its ability to deplete cells of growth-regulatory signaling proteins, such as proto-oncogene protein kinases and the steroid hormone receptors (Caplan 1999). GA interacts with the unique nucleotide-binding site of Hsp90, and blocks the conversion from its adenosine diphosphate (ADP)-dependent conformation to its adenosine triphosphate (ATP)-dependent conformation (Grenert et al 1997). Occupancy of this pocket by these compounds inhibits the chaperoning of Hsp90 protein substrates, and causes proteasome-dependent degradation of a select group of cellular proteins, including transmembrane tyrosine kinases, erbB family members (Miller et al 1994; Mimnaugh et al 1996), and insulin-like growth factor receptors (Sepp-Lorenzino et al 1995). GA exhibited antiproliferative effects on cells transformed with a number of oncogenes, particularly those encoding tyrosine kinases. It induced complete reversion of the transformed phenotype and differentiation in certain tumor cell lines (Uehara et al 1986; Vasilevskaya and O'Dwyer 1999). GA at nontoxic concentrations inhibited hormonal inducibility in several responsive cell systems. GA inhibited the binding of steroid hormones to their receptors, but did not interfere with the interaction of Hsp90 with the receptor polypeptide (Segnitz and Gehring 1997). In cell-free experiments, GA prevented the association of the p23 component with the Hsp90 heterocomplex system (Johnson and Toft 1995). GA blocked complete formation of Hsp90-immunophilin p23 complexes, and as a result intermediate progesterone receptor (PR) complexes dissociated and cycled back through the assembly pathway, bypassing the final maturation (Smith et al 1995). Interaction of GA and radicicol with Hsp90 destabilized the hormone receptors in a ligand-independent manner, leading to profound and prolonged depletion of their levels in breast cancer cells (Bagatell et al 2001).

The ligand-binding inhibitory effect of GA was investigated for the PR of the T-47D mammary carcinoma cells, the estrogen receptor (ER) of the MCF-7 mammary carcinoma cells, and the AR overexpressed in the fibroblasts (Segnitz and Gehring 1997). Hormone-binding ability of AR was found to be less sensitive than the other receptors to GA. It was found from experiments performed *in vitro* that Hsp90 is essential for hormone binding to the glucocorticoid receptor (GR) under all conditions studied (Bresnick et al 1989), although it is required for high-affinity hormone binding to the PR only at high temperatures (Smith 1993). Conflicting data exist for the hormone-binding ability of AR. Although several Hsps have been identified in AR complexes (Veldscholte et al 1992), it has been reported that Hsp90 is not essential for the maintenance of high-affinity hormone binding (Ohara-Nemoto et al 1991). These studies were performed with an N-terminal truncated form of the AR, and at low temperatures. Studies in yeast, using a mutant form of a gene

encoding Hsp90, indicated that Hsp90 maintains the AR in a high-affinity hormone-binding conformation (Fang et al 1996). In contrast to these studies, the use of the full-length AR from recombinant insect cells also suggested that chaperones do not play an essential role, at least at low temperatures (Xie et al 1992).

Androgen action mediated by AR plays an important role in the development of the normal prostate and even of prostate cancer. We, therefore, studied in detail the effect of GA on the stability and function of AR heterocomplexes and on the AR-regulated proteins in LNCaP prostate cancer cells using Hsp90-specific antibiotic GA. We show here that GA inhibits ligand-binding activity, enhances the degradation of the AR, and inhibits androgen-induced FKBP51 levels, resulting in loss of AR's functional activity in prostate cancer cells.

MATERIALS AND METHODS

Chemicals

GA was obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, USA), and was diluted in dimethylsulfoxide (DMSO) as a stock solution of 8 µg/mL. 17 α -Methyl-³H-mibolerone (³H-Mib) (80 Ci/mmol), ³⁵S-labeled methionine and cysteine, a trans label (1175 Ci/mmol), and unlabeled Mib were purchased from Dupont Research New England Nuclear (NEN) (Boston, MA, USA). Casodex ICI176334 was obtained from Zeneca Pharmaceuticals (Wilmington, DE, USA). Triamcinolone acetonide, cyclohexamide, and chloroquine were purchased from Sigma (St Louis, MO, USA). Anti-mouse AR antibody was obtained from Pharmingen (San Diego, CA, USA). Lactacystin (LC) was purchased from Alexis Biochemicals (San Diego, CA, USA).

Cell culture

The human prostate cancer cell line, LNCaP (American Type Culture Collection, Rockville, MD, USA), was grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum (FBS) at 37°C and 5% CO₂ until reaching approximately 50–70% confluence. The media were changed to serum-free RPMI 1640 to deplete undesired steroids for 24 hours prior to the experiments. Cells were then treated with 5% charcoal-stripped fetal bovine serum (CS-FBS) RPMI 1640 containing GA at designated concentrations with or without 1 nM of Mib, a nonmetabolizable synthetic androgen, which is equivalent to the physiological concentration of androgens (from NEN, dissolved in ethanol). Casodex a potent nonsteroidal antiandrogen was included in some experiments. An equivalent amount of the solvent was added to control cells.

Cell proliferation assay and immunoassay for PSA and hK2 quantitation

LNCaP cells were seeded at 2×10^4 cells/well in 24-well dishes. The cells were changed to serum-free and phenol red-free medium for 24 hours. The media were changed to 5% CS-FBS media and treated with GA at designated concentrations in the presence or absence of 1 nM Mib. Five days later, cell proliferation was measured by MTS assay method (Promega, Madison, WI, USA). A solution containing the tetrazolium compound [3-(4,5-dimethylthiazol)-2-yl-5-(3-carboxymeth-oxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS)] and an electron coupling reagent (phenazine methosulfate) were used in the assay (Cory et al 1991). MTS is bio-reduced by metabolically active cells into a formazan product and read at 490 nm. This absorbance is directly proportional to the number of living cells in the culture. Cells were incubated with freshly prepared, combined MTS-phenazine methosulphate (ratio of 20:1 by volume) solution for 1.5 hours at 37°C in a humidified 5% CO₂ atmosphere. PSA and hK2 levels in spent media were determined after 5 days of incubation by using the Tandem-E PSA kit or the Mayo hK2 assay (Hybritech Inc, San Diego, CA, USA). The results were normalized by cell density measured from the MTS assay.

Cell cycle arrest

Cell cycle analysis was performed on cells treated with 0.5 µM to 2.0 µM GA for 24 hours and 48 hours. Adherent and nonadherent cells were collected by centrifugation, washed with PBS, and fixed in 95% ethanol for 10 minutes on ice. The cells were pelleted, washed with PBS, and resuspended in 20 µg/mL propidium iodide in PBS containing 200 µM/mL RNase A. Samples were incubated for 1 hour at 37°C and subjected to fluorescence-activated cell sorter analysis (Becton Dickinson, Bedford, MA, USA).

Transient transfection assay

LNCaP cells were plated in 60-mm dishes until they reached a confluency of 50–70%. After depletion of steroids for 24 hours, cells were transfected with pGL3-basic luciferase vector (Promega) containing PSA 6-kb promoter (–5824/+12) (Zhu et al 1999) or with pGL3p containing 3 copies of an androgen-responsive element (ARE) from hK2 gene (Mitchell et al 2000), using liposomes containing dimethyldioctadecyl-ammonium bromide (DDAB) and L-lecithin (4:10 ratio) under serum-free conditions. The β-gal-CMV vector (pCMVB, Clontech Laboratories Inc, Palo Alto, CA, USA) was cotransfected to normalize for transfection efficiency. The parental vector pGL3p was used

as a control. After transfection, cells were treated with Mib, GA, or both, or vehicle for 24 hours in 5% charcoal-stripped FBS-RPMI 1640 culture medium. After incubation, cells were lysed, and luciferase activity was determined in cell lysates by use of the Lumate LB9507 luminometer using the luciferase assay kit (Promega) for the PSA promoter-luciferase or AR promoter-luciferase transfection. β-Galactosidase (β-gal) assay was performed for normalization purposes according to a published method (Hynes and Stern 1994). Three independent transfections were performed, and standard deviations were calculated.

Northern blot analysis

Cells were treated with varying amounts of GA and 1 nM Mib as indicated, and ribonucleic acid (RNA) was isolated by using Trizol reagent (GIBCO BRL, Rockville, MD, USA) according to the manufacturer's protocol. An RNA gel (1% agarose, 0.66 M formaldehyde in 3-[N-morpholine]propanesulfonic acid [MOPS] buffer) was run and transferred onto a nylon membrane, according to the GeneScreen protocol by NEN. Twenty micrograms of total RNA was loaded in each lane. Complementary DNAs (cDNAs) for AR, PSA, immunophilin, and glyceraldehyde-3-phosphate dehydrogenase were used as probes labeled with [³²P]deoxycytidine triphosphate (dCTP) by random priming and polymerase chain reaction labeling. Unincorporated nucleotides were removed by using a Sephadex G-10 spin column (Clontech). After denaturation, the labeled probes were hybridized with the RNA blots for 2 hours at 68°C using Express Hyb solution (Clontech). The hybridization was performed after prehybridizing the membrane for 4 hours with the same hybridization buffer containing 7% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.25 M sodium phosphate, pH 7.2. The membranes were washed with a washing solution containing 0.1× standard saline citrate (SSC) and 0.1% SDS. The membranes were autoradiographed at –70°C.

Immunoprecipitation and Western blot analysis

LNCaP cells were plated at a concentration of 9×10^5 cells/plate in 10-cm plates. The cells were serum starved for 24 hours in RPMI 1640 medium. For immunoprecipitation, cells were incubated with [³⁵S-labeled]-methionine and cysteine, a trans label (100 µCi) for 30 minutes at 37°C, and the cells were washed and treated with designated concentrations of GA in the presence of 1 nM Mib. After 2 hours, 4 hours, 6 hours and 8 hours, cells were harvested, and cell lysates were prepared by incubating cells in Triton X-100 lysis buffer (5×10^7 cells/mL) for 1 hour at 4°C. Nuclei were removed by centri-

fugation at $3000 \times g$, and the lysates were clarified by centrifugation at $10\,000 \times g$ for 1 hour, and protein concentrations were determined using a Bradford protein assay. Cell lysates were precleared by incubating with 10 μL of activated Sepharose per 200 μL supernatant, overnight, at 4°C , and centrifuged at $200 \times g$. Immunoprecipitations were performed with 3 mg of lysate and 3 μg of AR antibody bound to 20 μL of protein A-Sepharose. Immune complexes were washed with 1.0 mL of the following buffers. First and second wash was with dilution buffer (0.1% Triton X-100 and 0.1% bovine hemoglobin), third wash was with Tris-saline-azide solution (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 0.025% NaN_3), and fourth wash was with 0.05 M Tris-HCl (pH 6.8). After each wash, the complexes were centrifuged for 1 minute at $200 \times g$. Samples were incubated for 5 minutes at 100°C with $2\times$ SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels. The proteins separated were fixed by incubating the gel for 30 minutes in isopropanol-acetic acid-distilled water (25:10:65), and the gels were soaked and agitated in Amplify[®] (NAMP 100) for 30 minutes. After vacuum drying at 60°C , the gels were autoradiographed at -70°C .

For Western blot analysis, after incubating the cells for the desired time intervals, cells were washed with cold $1\times$ PBS once, and lysed with Radio immunoprecipitation assay (RIPA) buffer ($1\times$ PBS, 1% NP40 [Amaresco, Solon, Ohio, USA], 0.5% sodium deoxycholate, 0.1% SDS) or TENS buffer (Schulte et al 1997) for proteasomal degradation (50 mM Tris-HCl [pH 7.5], 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, 1% NP40) containing 20 $\mu\text{g}/\text{mL}$ aprotinin, 20 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 25 mM NaF, 25 mM β -glycerophosphate, and 5 mM *N*-ethyl maleimide (NEM). NP40 insoluble proteins were solubilized with SDS buffer (2% SDS, 100 mM dithiothreitol [DTT], 80 mM Tris [pH 6.8], 10% glycerol). Proteins were quantified by Bradford assay, and bovine serum albumin (BSA) was used as a standard (Sigma). Thirty micrograms of the protein was separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% nonfat, dry milk in TBST (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, and 0.1% Tween 20), overnight at 4°C , and subsequently incubated with a mouse antibody against human AR or β -tubulin (Sigma) for 1 hour at room temperature. The membranes were washed 3 times for 10 minutes each with TBST. Anti-mouse horseradish peroxidase (HRP) secondary antibody (Amersham, Piscataway, NJ, USA), used at a 1:10 000 dilution, was also incubated for 1 hour at room temperature. The membranes were washed again, and specific proteins were visualized with renaissance chemiluminescence (NEN). β -Tubulin was used as the protein loading and transfer efficiency control.

Assay of AR-binding affinity

The ^3H -Mib binding has been used extensively for the measurement of AR levels in various tissues (Syms et al 1985; Mukherjee et al 1999). The effect of GA on AR ligand binding was determined by competitive binding in the presence of the high-affinity AR ligand ^3H -Mib. AR-binding studies were performed by incubating the LNCaP cells with increasing concentrations (1–1000 nM) of cold Mib and with a saturating concentration of ^3H -Mib (1 nM at 37°C for 6 hours and 24 hours) in the presence or absence of 2 μM GA and proteasomal inhibitor LC (10 μM). The incubation mixtures also contained 1000 nM triamcinolone acetonide to block the interaction of Mib with PR. The plates were washed with PBS twice, and the cells harvested in PBS were centrifuged at 1000 rpm for 30 minutes. The bound ^3H -Mib was extracted with 400 μL of 95% ethanol for 30 minutes at room temperature. The supernatant was counted with 4.0 mL of scintillation cocktail. Specific binding was calculated as the difference between total binding and nonspecific binding with 1000 nM cold Mib.

RESULTS

Cell growth and cell cycle arrest

To evaluate the effects of GA on androgen-stimulated cell growth, LNCaP cells were treated with 1 nM Mib and various concentrations of GA. Cell growth was quantified by MTS assay 5 days later. GA inhibited the Mib-induced cell growth significantly (Fig 1). Increasing the concentration of GA from 2 μM to 4 μM gives no significant change in the cell growth. The decrease in cell growth was associated with cell cycle arrest in both G0-G1 and G2-M phases of 63.64% and 32.3% compared with the untreated controls of 57% and 24%, respectively. In addition, cells in the S phase were depleted when compared with the controls (4.0% vs 17%) (data not shown).

Inhibition of androgen-regulated gene expression

PSA and hK2 are widely used markers for AR-mediated gene expression in LNCaP cells (Mitchell et al 2000). In order to determine whether GA affects androgen-regulated gene activity, we treated LNCaP cells with 1 nM Mib plus GA (0.5 μM to 2.0 μM) for 5 days, then harvested the spent media for PSA and hK2 quantification. GA inhibited both PSA and hK2 secretion in a dose-dependent manner (Fig 2 A,B). To determine whether GA inhibits the expression of androgen-regulated genes at the transcriptional level, LNCaP cells were transfected with a luciferase plasmid construct, containing the PSA promoter or SV-40 minimal promoter with 3 copies of an hK2 ARE. After 24 hours of treatment with GA in the presence or absence of 1 nM of

Fig 1. The effect of geldanamycin (GA) on LNCaP cell growth. Human prostate cancer cell line LNCaP was grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum (FBS) at 37°C and 5% CO₂ until reaching approximately 50% confluence. The media were changed to serum-free RPMI 1640 to deplete undesired steroids for 24 hours prior to experiments. Cells were then treated with 5% charcoal-stripped FBS RPMI 1640 containing GA at designated concentrations with or without 1 nM of mibolerone (Mib). Equivalent amounts of solvent were added to control cells. Five days later, cell density was measured by MTS assay. The results were normalized by cell density. Four separate experiments are represented, and the error bars denote the standard deviation. * Indicates statistical significance over the Mib activity ($P < 0.05\%$).

Mib, cell extracts were prepared for luciferase assays. The results were normalized by β -gal activities. Mib-induced PSA promoter and hK2 ARE activities were greatly repressed by GA (Fig 2C). Quantitative evaluation showed a 6- to 10-fold increase in the promoter activity by Mib, which was completely inhibited by the drug. To check for reversibility of the inhibition in cells treated with Mib plus GA, the cells were transferred to GA-free media for 24 hours, and the results show that 50% of the PSA and hK2 promoter activities were regained. These results suggest that GA reversibly inhibited the AR-mediated transcriptional activity.

GA effect on AR, PSA, and immunophilin transcripts

It was shown that AR is autoregulated by androgens, which reduce AR messenger RNA (mRNA) but enhance AR protein levels in LNCaP cells (Tan et al 1988; Quarumby et al 1990; Krongard et al 1991). Northern blot analysis indicated that GA and Casodex reversed the androgen effect and increased AR mRNA levels (Fig 3). It should also be noted that in the absence of Mib, AR mRNA is increased by GA. We further looked at the effect of GA on androgen-regulated genes. Androgen-induced PSA mRNA levels were inhibited by GA and Casodex. We also

found for the first time that the expression of FKBP51, a large immunophilin known to exist in steroid receptor complex with Hsp90, was up-regulated by androgens. GA and Casodex markedly inhibited androgen-induced FKBP51 mRNA levels.

Effect of GA on AR and FKBP-51 protein levels

We further investigated whether GA could affect the protein levels of AR and immunophilin FKBP51. LNCaP cells were treated with 1 nM Mib and 2 μ M GA with or without the proteasomal inhibitor LC (10 μ M) for 15 minutes, 30 minutes, 6 hours, and 24 hours. LC was added 30 minutes prior to GA and Mib treatment. The inhibitory effects of GA on Mib-induced AR protein levels were very similar at 15 minutes and 30 minutes, and at 6 hours and 24 hours of incubation. The results of short-term (30 minutes) and long-term (24 hours) effects of GA are shown (Fig 4 A,B). In the presence of LC, the enhanced degradation of AR was protected, with accumulation of AR in the NP40 insoluble fraction. Densitometry of the AR levels in the NP40 soluble fraction after 24 hours of treatment revealed that GA reduced the AR levels to 15% of that in cells treated with androgen alone. Androgen-induced immunophilin FKBP51 protein levels were also drastically inhibited by incubation with GA for 6–48 hours (Fig 4C).

Degradation of AR by GA treatment

In order to measure the GA-induced degradation of AR, LNCaP cells were labeled with ³⁵S-methionine and cysteine. Stability of AR was determined by measuring the degradation of ³⁵S-labeled AR levels after GA treatment. The $t^{1/2}$ of the degradation of AR, in cells treated with Mib, was found to be around 6.5 hours (Fig 5). In cells treated with Mib along with GA, it was found to be around 2.5 hours. However, in cells treated with GA alone, the $t^{1/2}$ was 1.5 hours, and for untreated, control cells, it was 3.1 hours. GA treatment increased the degradation rate regardless of the presence or absence of Mib.

Ligand-binding characteristics of AR after GA treatment

We showed the regulatory effects of GA on AR protein levels as described previously. Next, we examined the ligand-binding ability of AR in GA-treated cells in the presence or absence of LC. A high level of ³H-Mib binding was observed with Mib alone and with Mib- and LC-treated cells (Fig 6). However, in cells treated with GA and LC in the presence of Mib, the specific ³H-Mib binding was negligible at all the time intervals studied. Al-

Fig 2. The effect of geldanamycin (GA) on the prostate specific antigen (PSA) and hK2 secretions and on the transcriptional activity in LNCaP cells. (A and B) After incubating the cells for 5 days as described in Figure 1, PSA and hK2 levels in the spent media were determined by using the Tandem-E PSA and hK2 kits. The results were normalized by cell density. (C) To assess the transcriptional activity, LNCaP cells in duplicate plates were transiently transfected with 6 kb PSA promoter and hK2 ARE constructs. Cells were then treated with 2 µM GA in the absence or presence of 1 nM of mibolerone (Mib) for 24 hours. Cell extracts were prepared and used for luciferase and β-galactosidase (β-gal) assay. "X" indicates reversibility of the promoter activity in which after 24 hours of Mib and GA treatment, GA was removed and the cells were incubated further for 24 hours with Mib and measured for luciferase-β-gal activities. β-Gal activity was used for transfection normalization. Four separate experiments are represented, and the error bars denote the standard deviation. * Indicates statistical significance over the Mib-activity ($P < 0.05\%$).

though the AR protein levels were detected in the NP40 insoluble fraction at 24 hours of treatment with GA and LC, as shown in the Western blot (Fig 4B), there was no detectable ^3H -Mib binding. These results suggest that the ligand-binding ability of AR could not be reversed by proteasomal inhibition because of the inhibition of Hsp90-chaperoning activity by GA.

DISCUSSION

Hsp90 has been shown to be important for the high-affinity ligand-binding activity of GR and PR (Bamberger et al 1997; Liu and DeFranco 1999). Previous studies showed that the ligand-binding affinity of AR is not largely dependent on Hsp90 interaction (Ohara-Nemoto et al 1991; Segnitz and Gehring 1997). In this report, we have studied the effect of GA via Hsp90 on the expression

and function of AR in LNCaP prostate cancer cells. The inhibitory effect of GA on LNCaP cell growth was found to be associated with cell cycle arrest. In the human leukemia HL-60 cells, GA inhibited G1-S transition and caused the G2-M phase of cell cycle arrest (Yamaji et al 1995). PSA and hK2 secretions were almost completely inhibited by treatment with GA. This indicates the complete loss of transactivation of AR with GA treatment. Our finding that GA destabilized AR protein and induced a reduction in the cellular receptor levels is similar to the previously reported results with GR and PR (Smith et al 1995; Bamberger et al 1997). These results show that treatment with GA blocks androgen-induced gene activation in a reporter system, and fit well with the recent findings that treatment with GA and radicicol inhibited androgenic stimulation of the ARE of cysteine-rich secretory proteins (Haendler et al 2001). The return of the

Fig 3. Northern blot analysis of the messenger ribonucleic acid (mRNA) levels in LNCaP cells treated with 2 μ M GA and 5 μ M Casodex in the absence or presence of 1 nM mibolerone (Mib). After 24 hours, total RNA was prepared, and Northern blot analysis performed using [32 P]-labeled probes. Human androgen receptor (483 bp, 2844–3326 from ATG), prostate specific antigen oligonucleotide (77 bases, 1159–1235 from ATG), FKBP52 (705 bp, 1–705 from ATG) complementary deoxyribonucleic acids (cDNAs) were used as probes, and glyceraldehyde-3-phosphate dehydrogenase (450 bp) cDNA was used as the control of RNA loading and transfer efficiency.

function of AR after removal by GA, as measured by the PSA promoter activity, may be partly because of the stabilization of receptor levels or the synthesis of new receptors.

Previously, the inhibitory effects of GA on the AR over-expressed in fibroblasts were investigated (Segnitz and Gehring 1997). Ligand-binding activities of the PR and ER were completely inhibited, whereas AR showed only 33% inhibition. Thus, compared with other steroid receptors, AR was much less sensitive to GA. Another study with truncated ARs expressed in *E coli* or in vitro translated with rabbit reticulocyte lysates showed that there is no absolute requirement of Hsp90 association for hormone binding to the AR (Nemoto et al 1992). However, we showed that GA treatment of LNCaP prostate cancer cells caused a decrease in both endogenous AR protein levels and ligand-binding activity with functional impairment of AR, suggesting that Hsp90 is essential for AR activity in prostate cells.

The FK506-binding proteins, FKBP51, FKBP52, CyP40, and protein phosphatase (PP5) were identified as members of PR, GR, and ER complexes (Pratt and Toft 1997). The presence of FKBP51 in GR heterocomplexes has been linked to reduced GR-binding affinity and glucocorticoid resistance in the squirrel monkey (Denny et al 2000). In addition, there is an emerging picture of a wider role for

Fig 4. Western blot analysis of the protein levels of androgen receptor (AR) and immunophilin FKBP51 in LNCaP cells treated with geldanamycin (GA) and 1 nM mibolerone (Mib) in the presence or absence of proteasomal inhibitor LC (10 μ M). (A) After 30 minutes and (B) 24 hours of treatment with GA, cells were lysed in TENS buffer (1% NP40). Insoluble precipitates were resolubilized with gel-loading buffer (2% sodium dodecyl sulfate). Equal amounts of protein were analyzed by Western blotting with monoclonal antibody against AR (1:2000). "S" and "IS" indicate NP40 soluble and insoluble fractions. (C) After incubation for 6–48 hours, cell extracts were prepared, and 30 μ g of the protein were analyzed by Western blot using monoclonal antibody against FKBP51 (1:5000 dilution). β -Tubulin is used as a control of protein loading and transfer efficiency. The experiments were repeated thrice, and the representative blot was shown.

Fig 5. Degradation rate of androgen receptor in LNCaP cells treated with geldanamycin (GA) in the presence or absence of mibolerone (Mib). After 24 hours of incubation of cells in serum-free Roswell Park Memorial Institute (RPMI) 1640 media, the cells were placed in RPMI 1640 media with charcoal-stripped serum and without methionine, cystine, and L-glutamine for 20 minutes, and incubated with 100 μ Ci of 35 S-labeled-methionine and cysteine for 30 minutes. The cells were washed and incubated with 1 nM Mib and 2 μ M GA. This was followed by a chase in the absence of labeled methionine and cysteine for increasing times as indicated. Androgen receptor (AR) was immunoprecipitated and analysed on the sodium dodecyl sulfate gel. Autoradiographic signals were quantitated using densitometric scanning. Degradation half-times of AR at 37°C were calculated. Data represents the means of duplicate determinations.

the steroid receptor-associated immunophilins in cellular functions. They appear to be involved in cell cycle regulation, progression, and signal transduction (Doucet-Brunin et al 1995; Yeh et al 1995; Duina et al 1996). It was previously shown that immunophilins are overexpressed in breast tumors compared with normal tissues (Ward et al 2000). We sought to determine the levels of FKBP51 in prostate cancer cells. In this study, we provide the first evidence that FKBP51 levels are significantly induced by androgens at both mRNA and protein levels. GA and Casodex were found to inhibit the androgen-induced FKBP levels. Recently, we have reported the inhibition of Mib-induced expression of FKBP51 mRNA levels by docosahexaenoic acid in LNCaP cells (Chung et al 2001). These results are in agreement with previous observations that showed up-regulation of FKBP51 protein levels by progesterin and dexamethasone (Kester et al 1997).

Earlier studies have shown that GA enhanced degradation of several tyrosine kinases, IGF-1 receptor, nNOS, GR, p185erb2, and mutant p53, and this degradation is mediated by the proteasome pathway (Dasgupta and Momand 1997; Schulte et al 1997; Whitesell et al 1997; Bender et al 2000). We showed that GA enhances the deg-

Fig 6. Effect of geldanamycin (GA) on the ligand binding of androgen receptor (AR). Competitive binding of unlabeled mibolerone (Mib) for 3 H-Mib with AR in LNCaP cells. Cells were plated at 3.75×10^5 cells/plate in 6-cm plates in charcoal-stripped fetal bovine serum Roswell Park Memorial Institute 1640 medium until reaching approximately 50–60% confluence. After depleting undesired steroids, as mentioned earlier, cells were treated with 10 μ M lactacystin, with 2 μ M GA, and in the presence of 1 nM 3 H-Mib with or without unlabeled Mib. Unlabeled ligand concentration ranged from 1 nM to 100 nM as indicated, and incubations were for (A) 6 hours, and (B) 24 hours at 37°C in the presence of 1 nM 3 H-Mib. Nonspecific binding was accounted for by using parallel incubations with 1000-fold excess unlabeled Mib. Data are expressed as percentage inhibition of binding observed in the absence of competing ligands. The figure represents data obtained from a single series of experiments that have been repeated thrice.

radiation rate of AR. In the presence of Mib and GA, the degradation was faster compared with that of cells without any treatment. LC is a permeable, irreversible proteasomal inhibitor. Treatment with LC and GA caused the accumulation of AR in the NP40 insoluble pellet without restoring the hormone-binding ability. This is quite similar to the GA effect on p185^{c-erbB2} receptor protein-tyrosine kinase, where it induced proteasomal degradation of the receptor (Mimnaugh et al 1996).

In summary, we showed that AR is a highly sensitive target of GA exposure, leading to rapid proteolytic turnover and complete loss of ligand-binding ability as well as androgen-dependent transactivation function of AR. The half-life of AR decreased significantly, indicating that Hsp90 is essential for stability and ligand-binding activity of AR. These results, therefore, demonstrate that Hsp90 is essential for the maintenance of the functionality of the AR in prostate cancer cells. Similar to the GR and Raf-1, the GA-mediated increased degradation of AR seems to be dependent on the proteasome pathway. Additionally, we also observed androgen-induced regulation of FKBP51 levels. These studies provide strong evidence that GA exhibits a high potency of modulating androgen action by blocking both AR expression and ligand-binding activity in prostate cancer cells, indicating that GA may be a candidate of choice for prostate cancer intervention.

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