Mechanism of Antiandrogen Action: Key Role of Hsp90 in Conformational Change and Transcriptional Activity of the Androgen Receptor†

Virginie Georget,‡,§ Béatrice Térouanne,‡ Jean-Claude Nicolas,‡ and Charles Sultan*,‡,||

Pathologie Moléculaire des Récepteurs Nucléaires, Institut National de la Santé et de la Recherche Médicale, INSERM U439, 70 Rue de Navacelles, 34090 Montpellier, France, and Laboratoire d'Hormonologie, Hôpital Lapeyronie, CHU, Montpellier, France

Received April 3, 2002; Revised Manuscript Received July 8, 2002

ABSTRACT: Activation of the androgen receptor (AR) is induced by ligand binding through conformational changes leading to control of gene expression. Antiandrogens compete with androgens for AR occupancy and subsequently block at least one step in AR action. Analysis of nuclear transfer kinetics using the GFP-AR fusion protein and partial proteolysis analysis provided evidence that the ligand-bound receptor was in equilibrium between at least two distinct conformations, leading to the production of 35 and 29 kDa trypsin-resistant fragments. It also indicated that this equilibrium may regulate the rate of nuclear transfer. The slowing of nuclear transfer by antiandrogens was correlated with the amount of receptor in conformation leading to the 35 kDa trypsin-resistant fragment. To establish the role of heat shock protein (hsp) 90 activity in antiandrogenic action, the effect of geldanamycin (GA) was evaluated in both in vitro assays and live cells. We demonstrated that in vitro hsp90s are required to stabilize the receptor in the inactive conformation and that hsp90 activity is involved in the integrity and nuclear transfer of agonist- and antagonist-bound AR. Furthermore, nuclear transfer is not the only step affected by GA since this compound was also active on a constitutively nuclear AR (GFP-NLS-AR). Hsp90 inactivation impedes interaction of androgen-bound GFP-NLS-AR with nuclear components and inhibits transcriptional activity. We conclude that hsp90s are required for the acquisition of active conformation in agonist-bound AR to regulate nuclear transfer, nuclear matrix binding, and transcriptional activity. Pure antiandrogens block the transconformational change of AR in an intermediary complex unable to acquire the active conformation and to dissociate the hsp90.

Antiandrogens are used in the management of androgen-dependent diseases such as hirsutism, acne, and prostate cancer. Besides their therapeutic usefulness, the elucidation of their mechanisms of action is of great interest in understanding the molecular action of the androgen receptor (AR).

AR belongs to the superfamily of nuclear receptors whose members are ligand-responsive transcription factors that possess marked structural and functional similarities (*1*). It is composed of four domains: the amino-terminal transcription activation domain, which possesses a ligand-independent transcriptional function (AF-1); the DNA-binding domain, which interacts with a specific DNA sequence named androgen-responsive element; the hinge region, which includes a part of the nuclear localization signal; and the carboxy-terminal ligand-binding domain (LBD), also involved in the dimerization process and which has a ligand-dependent transcriptional activity (AF-2) (*2*). Unliganded AR is located in the cytoplasm and is associated in an 8−9S heterocomplex with several heat shock proteins (hsp) and different immunophilins (for review, see refs *3*−*5*). Hsp90 interacts with the AR LBD and participates in the activation process by maintaining apoAR in a high-affinity ligand-binding conformation. Androgen binding induces its nuclear transport followed by interaction as a homodimer with the target DNA sequences, inducing recruitment of downstream transcription factors and the subsequent transcription of target genes.

Several mechanisms have been proposed to explain the action of androgen antagonists on AR. These include impaired dissociation of hsp, receptor dimerization, nuclear translocation, DNA binding, and abrogation of coactivator binding and promotion of corepressor interaction (*6*). In previous works (*7*, *8*), we studied the intracellular dynamics of AR using a GFP-AR fusion protein and described that antiandrogens could decrease the rate of AR nuclear transfer. Limited proteolysis assays differentiated two states of AR, an active state induced by androgen corresponding to a 29 kDa resistant band and an inactive state acquired after antiandrogen treatment leading to a 35 kDa proteolytic fragment (*9*, *10*). Kuil et al. (*11*) reported that the release of hsp from the heterocomplex, unmasking a new trypsin site, corresponds to a shift from the 35 to the 29 kDa band. The stabilization of AR in an inactive state by pure antiandrogens could be explained by the nonrelease of hsps from the

† Part of this work was supported by the Institut National de la Santé et de la Recherche (INSERM) and the Association pour la Recherche sur le Cancer (ARC 5205, 1999).

* Corresponding author. Tel: (+33) 467 33 86 96. Fax: (+33) 467 33 83 27. E-mail: chsultan@u439.montp.inserm.fr.

‡ Institut National de la Santé et de la Recherche Médicale, INSERM U439.

§ Permanent address: Bastiaens Group, EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

|| Laboratoire d'Hormonologie, Hôpital Lapeyronie.

10.1021/bi0259150 CCC: $22.00
Published on Web 09/04/2002

heterocomplex. The relationship between the stabilized conformations and the intracellular dynamics of AR induced by antiandrogens suggests a role of hsp90 activity in their mechanisms.

To define this role, we explored hsp90 activity in the AR acquisition of active conformation and nuclear transfer. To analyze hsp90 activity, geldanamycin (GA) was applied both in vitro by proteolysis assays and in the cell using the GFP-AR fusion protein. GA is a benzoquinone ansamycin antibiotic that has been found to bind in a pharmacologically specific manner to hsp90 (*12*) and to disrupt its function (*13*). GA treatment of cells resulted in a complete loss of p23 protein association in the heterocomplex of GR (*12*). Exposure of intact cells to GA inhibited the hormone-binding abilities of androgen, glucocorticoid, progestin, and estrogen receptors (*14*). The transcriptional activity (*12*, *14*, *15*) and nuclear transport (*13*, *16*) of GR were also inhibited by GA. In the present study, we report that GA affects the nuclear translocation of AR by impeding the action of hsp90, which is required not only to stabilize inactive AR (aporeceptor or receptor bound to antagonist) but also to acquire active conformation after agonist binding.

MATERIALS AND METHODS

Chemicals. R1881 (methyltrienolone) was purchased from NEN Life Science Products (Paris, France). RU23908 (nilutamide) was a gift from Roussel-UCLAF (Romainville, France); cyproterone acetate was purchased from Sigma (Saint-Quentin Fallavier, France). Hydroxyflutamide and ICI176344 (bicalutamide) were obtained from Theramex (Monaco). Geldanamycin was purchased from Life Technologies (Cergy-Pontoise, France). Trypsin was obtained from Sigma.

Plasmids. We constructed the pGFP-AR plasmid, which contained the GFP cDNA bearing the S65T mutation (Clontech, Palo Alto, CA) fused to the 5′ end of the full-length AR cDNA. Briefly, we inserted the 2−36 amino acid residues of AR lacking in the previously described p-C1-GFP-AR (*7*). For this, we amplified by polymerase chain reaction the 3−349 bp of AR cDNA with primer sense 5′ CGAAGATCTCGAGAAGTGCAGTTA 3′ and primer antisense 5′ CGACACTTCCAACGACAAGGAG 3′ using the pCMV5-hAR as template [gift from Dr. T. Brown (*17*)]. The *Xho*I restriction site was inserted before the AR gene in the 5′ primer. The generated amplified product was digested by *Xho*I and *Xma*I enzymes and inserted at the cognate sites into the p-C1-GFP-AR (*7*).

The plasmid pGFP-NLS-AR was obtained by inserting into pGFP-AR three copies of the nuclear localization signal (NLS) of SV40 large T antigen. pECFP-Nuc (Clontech, Palo Alto, CA) was digested by *Bgl*II and *Bam*HI enzymes to isolate the tripartite NLS. The resulting fragment was inserted into the pGFP-AR digested by *Bgl*II.

pSG5-hAR contained the full-length cDNA of human AR. The p mouse mammary tumor virus luciferase (MMTV-luc) reporter plasmid was kindly provided by Dr. H. Richard-Foy (*18*).

Transcriptional Activity Assays. CV-1 cells were transfected in 12-well dishes with 0.15 μg of pGFP-AR or pGFP-NLS-AR, 1 μg of p mouse mammary tumor virus luciferase (MMTV-luc) used as the androgen-regulated gene, and 0.25 μg of pCMV-β-galactosidase. Twelve hours after transfection, the cells were incubated with hormone. Cells were then harvested in lysis buffer: 25 mM Tris−H_3PO_4 (pH 7.8), 2 mM CDTA, 1% Triton X-100, and 10% glycerol. Aliquots were used in the β-galactosidase activity assay. Luciferase activity was measured by the reaction of lysate with the luciferin solution: 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, 20 mM Tricine (pH 7.8), 1.07 mM $(MgCO_3)_4$-$Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, and 1 mM EDTA, using a luminometer (LKB Instruments, Rockville, MD). Each incubation was performed in duplicate, and experiments were performed in triplicate.

Limited Proteolytic Digestion of in Vitro Produced Receptors. Expression plasmid (pSG5-hAR) was transcribed and translated with the TNT^{T7}-coupled reticulocyte lysate system (Promega, Charbonieres, France) in the presence of [^{35}S]-methionine (1000 Ci/mmol; ICN, Orsay, France) for 2 h at 30 °C, according to the manufacturer's instructions.

Five microliters of labeled translation mix was preincubated at 37 °C with 0.5 μL of vehicle or ligand previously diluted in PEG 300 [50% (v/v)] to a final concentration of 1 or 100 nM for R1881 and 10 nM or 10 μM for antiandrogens. For limited proteolytic digestion, 5 μL of trypsin (diluted in water) was added to the preincubation mix, giving final trypsin concentrations of 25 and 50 μg/mL. Incubations were conducted for 10 min at room temperature and stopped by cooling on ice and addition of 10 μL of SDS sample buffer. Samples were boiled for 10 min, and 10 μL was loaded onto 0.75 mm thick 12% SDS−polyacrylamide gel. After electrophoresis, the gels were fixed in methanol (30%, v/v)−acetic acid (10%, v/v), treated with Entisify (Dupont de Nemours, Brussels, Belgium), and vacuum-dried at 80 °C for 20 min. Gels were exposed to a Fujix film imaging plate for 1 h and to autoradiography overnight. The intensity of the bands were semiquantified using the Fujix software. The results are represented as a ratio between the protected band after trypsic action and the nondigested receptor.

Kinetics of Androgen Receptor Nuclear Translocation. COS-7 cells were transfected on 2 × 2 cm^2 Lab-Tek chamber slides (Nunc Inc., Naperville, IL) with 1 μg of pGFP-AR. Twenty-four hours after transfection and before nuclear transfer kinetics, the cells were cultured at 30 °C for at least 30 h. Mammalian cells expressing GFP-S65T exhibit stronger fluorescence when grown at temperatures lower than 37 °C (*19*). The living cells were observed directly on the chamber slide using an inverted fluorescence microscope (Diaphot 200; Nikon, Champigny-sur-Marne, France) with a FITC filter. This microscope was coupled to a CCD camera (Night Owl; EGG-Berthold, Evry, France) to record cells. The cells were first observed without hormone and after incubation at 37 °C in different conditions. Thus, the same living cell was analyzed and recorded at different times. The kinetics of nuclear transfer were studied at 37 °C. Each image was analyzed, and the signal intensity of cytoplasmic and nuclear areas was quantified using the IPLab software. The percentages of nuclear fluorescence compared to the total fluorescence were calculated and plotted on the time scale.

RESULTS

Comparison of Antiandrogen Effects on AR Conformation and Time-Lapse Imaging of GFP-AR Dynamics. The effects

of antiandrogens were compared intraassay regarding two parameters: the induced conformation by in vitro limited proteolysis assay and the dynamics of AR in transfected cells.

AR synthesized in vitro in the presence of [^{35}S]methionine was incubated with various ligands prior to trypsic digestion. As previously described (*10*, *11*), AR was completely digested by 50 μg/mL trypsin while a 29 kDa proteolysis-resisting band resulted from R1881-bound AR trypsin digestion (1 and 100 nM) (Figure 1A). Cyproterone acetate (10 nM) stabilized the receptor in a conformation providing a major 35 kDa resistant form while a 29 kDa band appeared at higher concentration. Proteolytic digestion of AR incubated with bicalutamide, hydroxyflutamide, or nilutamide resulted in a major 35 kDa resistant fragment.

Kinetics studies were performed in COS-7 cells transfected by pGFP-AR to define the capacity of antiandrogens to alter the cytoplasmic−nuclear transfer of AR induced by androgen. GFP-AR was predominantly localized in the cytoplasm in the absence of hormone, as previously reported (*8*, *20*). Cells were then incubated at 37 °C simultaneously with R1881 (1 nM) and an antagonist (1 μM) for competition analysis. The kinetics were quantified and compared to the basal androgen-dependent nuclear transfer with R1881 (1 nM). All tested antiandrogens had an inhibitory effect on the AR nuclear import generated by R1881 binding (Figure 1B,C). These kinetics are representative of numerous time-lapse imagings performed in the same conditions. Cyproterone acetate (1 μM) slightly slowed the transfer induced by 1 nM R1881. The maximal transfer of GFP-AR was observed after 120 min of incubation with R1881 while cyproterone acetate required 180 min of incubation. In the cells incubated with hydroxyflutamide in the presence of R1881, the kinetics of AR nuclear transfer were partially inhibited. Nilutamide and bicalutamide drastically slowed the nuclear transfer, and only slight nuclear transfer of AR was observed after 3 h of incubation. However, AR was nuclear after 24 h with all tested antiandrogens (data not shown).

These data show that antiandrogens induced a different conformation than that induced by agonists and that transfer rate seems to be correlated with the equilibrium between active and inactive conformations.

Role of Hsp90 in the Acquisition of Conformational Change. To elucidate the role of hsp90 in the conformational change of AR, we analyzed the effect of geldanamycin (GA) on the proteolysis of AR. The active effect of GA in reticulocyte lysate is demonstrated in Figure 2A. In vitro produced AR, incubated at 37 °C first with GA (10 μg/mL) (step 1) and next with R1881 (100 nM) (step 2), did not acquire the active conformation and was significantly digested (Figure 2A). This impairment of AR protection could be linked to the decrease in androgen-binding affinity by GA previously described in cellular androgen-binding assays (*14*). To evaluate the effect of hsp90 inactivation on AR transconformation, the in vitro produced AR was then stimulated on ice with R1881 to form steroid−receptor complexes without dissociation of hsp90, followed by treatment on ice with or without GA. In a third step, the incubation was achieved at 37 °C to permit receptor transformation. This experimental procedure was previously described by Czar et al. for cellular analysis of glucocorticoid receptor dynamics (*13*). In the presence of agonist, an expected band of 29 kDa was obtained after proteolysis despite the presence of GA (Figure 2B); however, the calculated band intensity was lower than in the absence of GA, suggesting that conformational change of ligand-bound AR could be partially dependent on hsp90 activity.

In vitro produced AR was also incubated with androgen or antiandrogens for 30 min at 37 °C followed by an incubation with DMSO or GA at 37 °C (Figure 2C). Addition of GA after cyproterone acetate binding provided the complete proteolytic digestion of the 35 kDa band. The 29 kDa band was less intense than in the absence of GA, reflecting the equilibrium between the two conformations **(**Figure 2C, lower panel). The 35 kDa band observed after nilutamide binding disappeared after incubation with GA. The AR bound to nilutamide was not protected in the presence of GA. These results show that only the active conformation of AR leading to the 29 kDa band was stable in the presence of GA. AR in its inactive conformation needs hsp90 to be protected and to produce a 35 kDa resistant band. Pure antiandrogens could block AR in an intermediary complex formed during AR activation and stabilized by hsp90.

Role of Hsp90 in AR Nuclear Transfer. To define the role of hsp90 in AR nuclear transfer and its potential interference with antiandrogen action, we explored the effect of GA on the GFP-AR cellular dynamics in living cells.

Cells were treated in experimental conditions identical to those described above for limited proteolysis assays. GA (1 μg/mL) incubation for 30 min at 37 °C to cells caused distribution of GFP-AR in numerous cytoplasmic aggregates (Figure 3A). Addition of GA for 30 min at 37 °C after 3 h of incubation with R1881 had no visible effect on the subnuclear localization of GFP-AR. In both conditions, the fluorescence appeared to be accumulated in discrete clusters. Last, cells were preincubated for 30 min on ice, and then with R1881 (100 nM) for 30 min on ice, to allow ligand binding without dissociation of hsp90. GA was then added for 30 min prior to shifting the temperature to 37 °C to permit receptor transformation and translocation to the nucleus. These experimental conditions were appropriate for efficient transport since AR was nuclear in the absence of GA. Conversely, addition of GA after androgen binding on ice partially impaired nuclear transfer of GFP-AR in numerous cells presenting reticular patterns and aggregates of GFP-AR in the cytoplasm (Figure 3A). Aggregate formation was observed in cells expressing various levels of GFP-AR.

Regarding the effect of antiandrogen binding, cells were incubated for 3 h at 37 °C with an antiandrogen (1 μM) and then with GA (1 μg/mL) for 30 min at 37 °C (Figure 3B). In cells preincubated with cyproterone acetate, GFP-AR was predominantly nuclear with or without GA, and only a few aggregates were observed in the cytoplasm after GA treatment. GA induced the formation of abundant cytoplasmic aggregates in cells preincubated with hydroxyflutamide, bicalutamide, or nilutamide (Figure 3B). This effect was visualized in 28%, 36%, and 38% of fluorescent cells, respectively. These results (Figure 3) indicate that interactions of hsp90 or associated proteins with unbound AR and with agonist- or antagonist-bound AR in the cytoplasm were required for AR integrity and its nuclear transfer.

Role of Hsp90 on a Constitutively Nuclear AR. We suggest then that agonist- or antagonist-bound AR was transported into the nucleus complexed to hsp90. To investigate the

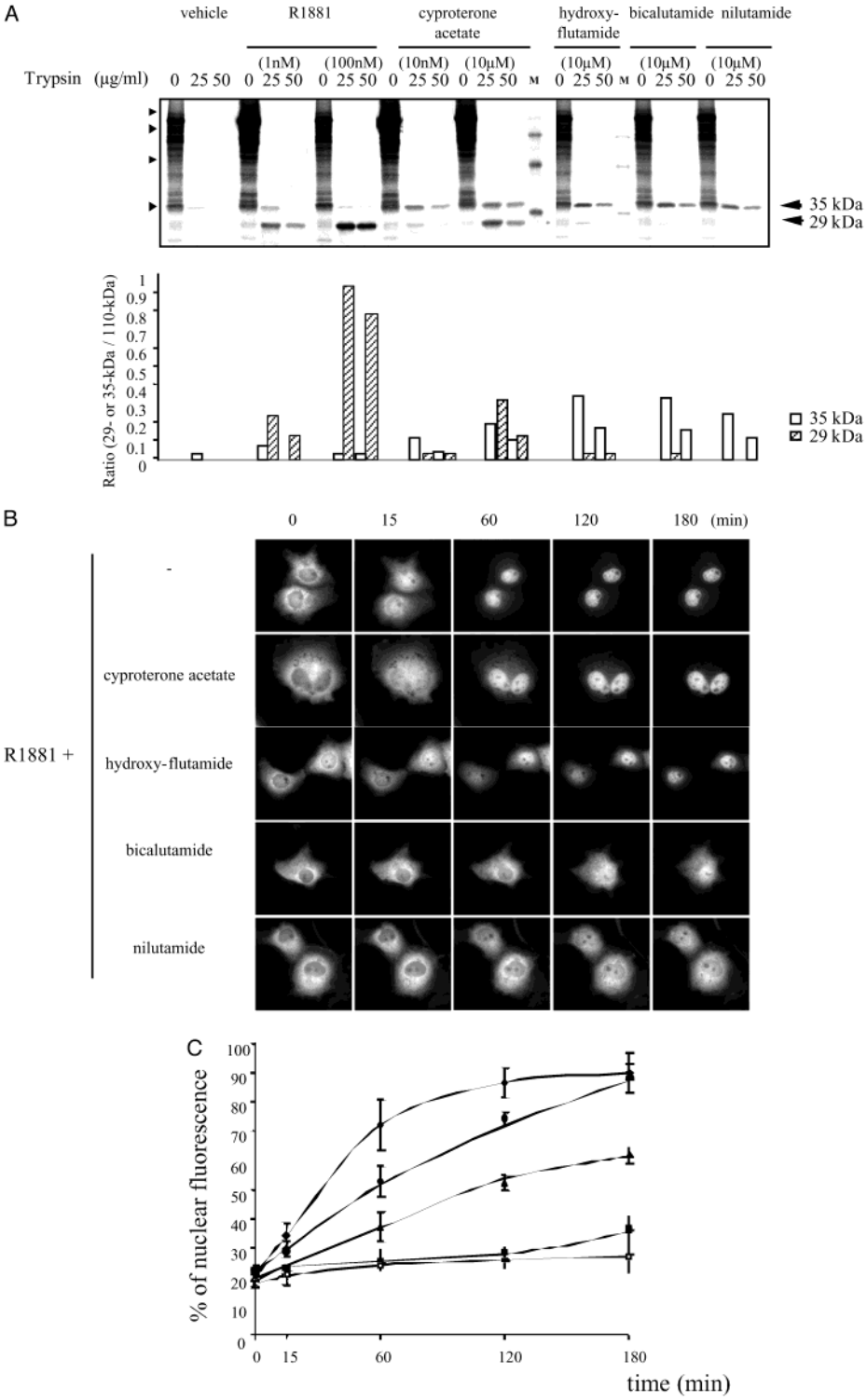

FIGURE 1: Antiandrogenic effect on conformational change and androgen-dependent nuclear transfer of AR. The tested antiandrogens are classified as steroidal antiandrogens (cyproterone acetate) and nonsteroidal antiandrogens [hydroxyflutamide, bicalutamide (or casodex), and nilutamide (or anandron)]. Part A: In vitro produced AR was incubated at 37 °C with hormone or antihormones prior to addition of different concentrations of trypsin (25 and 50 μg/mL). The digested products were subjected to SDS−PAGE electrophoresis. The ^{14}C-labeled protein mass marker (M) migrated as bands of 97.4, 66, 46, and 30 kDa. Gels were exposed to a Fujix film imaging plate for 1 h. On the lower panel are represented the relative intensities of the bands by calculation of the ratio between the protected band (35 or 29 kDa) and the nondigested receptor (110 kDa) incubated in each hormonal treatment. Part B: COS-7 cells transiently transfected with GFP-AR were observed directly with an epifluorescence microscope coupled to a CCD camera to record cells maintained at 37 °C during the kinetic studies. Cells were incubated at 37 °C in the presence of R1881 (1 nM) alone or associated with antiandrogen (1 μM). The cells were recorded before incubation and after 15, 60, 120, and 180 min of treatment. The dynamics obtained with antiandrogens were compared with the nuclear import kinetics of GFP-AR induced by R1881 (1 nM). Part C: Images obtained from the different kinetics as in part B are analyzed by IPLab software. The intensities of fluorescence are quantified for the nuclear and cellular areas. The percentage of nuclear fluorescence for each cell as a function of time is calculated. Key: (◆) R1881, 1 nM; (●) cyproterone acetate, 1 μM, + R1881, 1 nM; (▲) hydroxyflutamide, 1 μM, + R1881, 1 nM; (■) bicalutamide, 1 μM, + R1881, 1 nM; (□) nilutamide, 1 μM, + R1881, 1 nM.

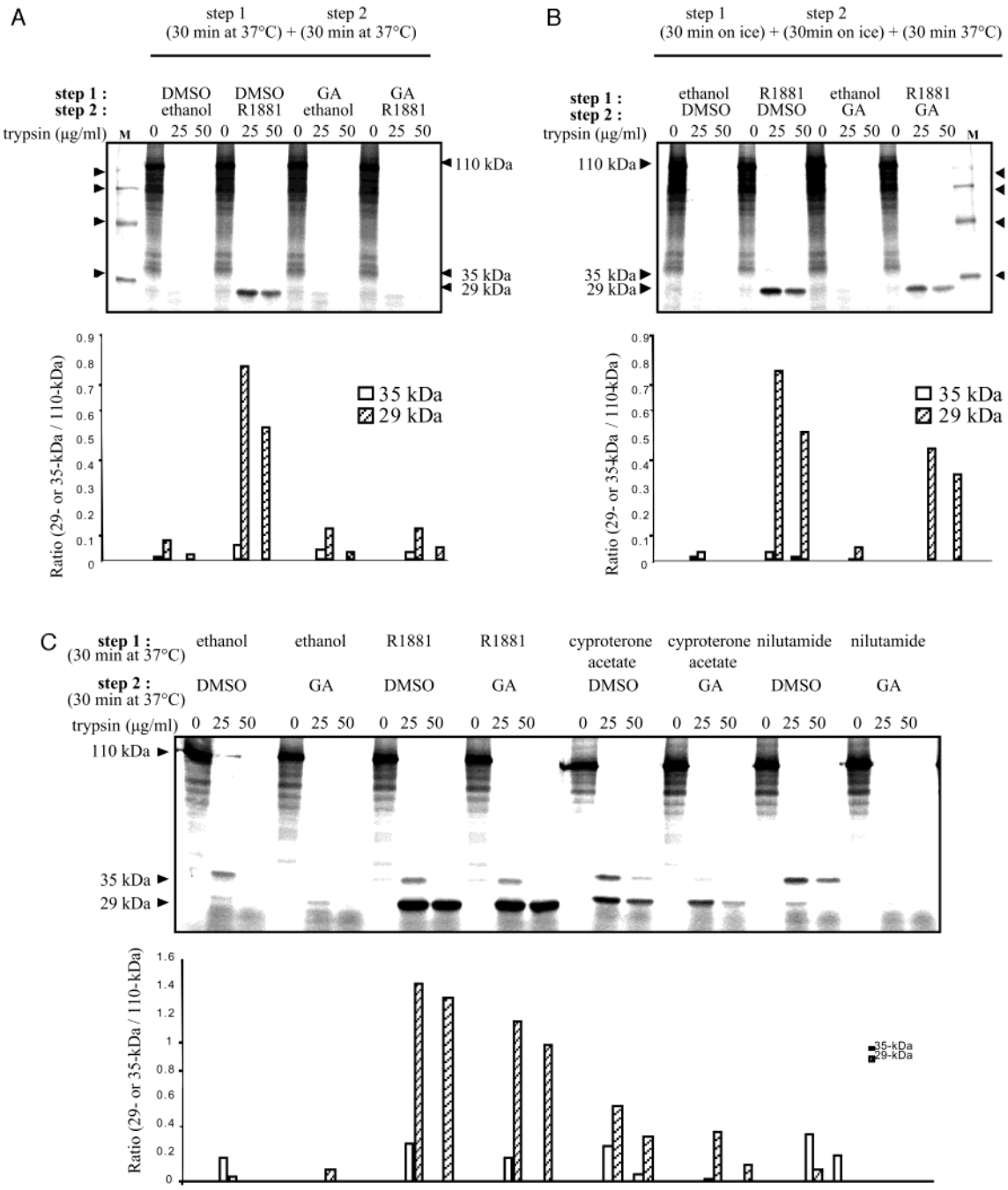

FIGURE 2: Effect of geldanamycin (GA) on the conformational change in AR. The AR produced in the reticulocyte lysate in the presence of [^{35}S]methionine was incubated at various temperatures with DMSO or GA and ethanol, hormone, or antihormones. The binding product was digested at room temperature by 25 and 50 μg/mL trypsin and analyzed by SDS−PAGE and autoradiography. Part A: AR was first incubated at 37 °C with DMSO or GA (10 μg/mL) and then with ethanol or R1881 (0.1 μM) before trypsic digestion. Part B: In vitro produced AR was incubated with ethanol or R1881 (0.1 μM) for step 1, and DMSO or GA (10 μg/mL) was added for step 2. Steps 1 and 2 were performed on ice and followed by an incubation at 37 °C for 30 min. Part C: In vitro produced AR was incubated with ethanol, R1881 (0.1 μM), or various antiandrogens (10 μM) for 30 min at 37 °C (step 1); GA (10 μg/mL) or DMSO was then added for 30 min at 37 °C (step 2). Gels were exposed to a Fujix film imaging plate for 1 h, and the intensities of the bands were measured with the Fujix software. The lower panels represent the relative values of the protected bands compared to nondigested receptor.

activity of hsp90 in the nucleus, we constructed a fusion protein with three repeated nuclear localization signals (NLS) between GFP and AR (Figure 4A). The correct expression of GFP-NLS-AR in COS-7 transfected cells was verified by immunoblotting and immunocytochemistry (data not shown). As expected, the microscopic analysis of transfected COS-7 cells revealed a predominant nuclear localization of GFP-NLS-AR in its unliganded form (Figure 4B). The three copies of constitutive NLS were functional in the fusion protein and were able to address the AR to the nucleus in the absence of hormone (80% of total fluorescence). After 30 min of incubation with R1881, we observed formation of nuclear foci, and the remaining cytoplasmic fluorescence was translocated into the nucleus. Intranuclear distribution of GFP-NLS-AR and GFP-AR bound to nilutamide was homogeneous with no cluster formation. Figure 4C shows that GFP-NLS-AR was able to transactivate an androgen-regulated reporter gene in the presence of R1881 with an EC-50 identical to that of GFP-AR. However, we noted a reduction in the induction factor. The antiandrogen nilutamide, as well

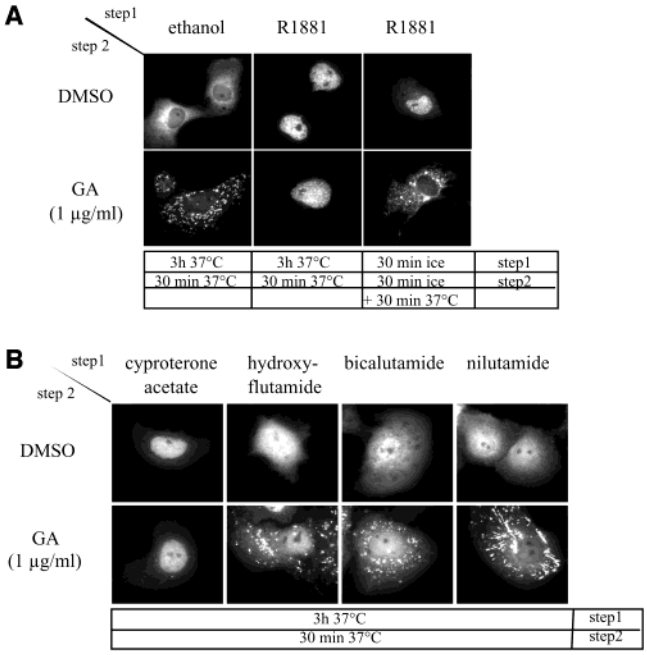

FIGURE 3: Effect of geldanamycin (GA) on nuclear transfer of AR. Part A: COS-7 cells expressing GFP-AR were incubated in various conditions with vehicle or R1881 (0.1 μM) for step 1 followed by incubation with vehicle or GA (1 μg/mL). Time of incubation and temperature are indicated for each case. After treatment, living cells were directly observed by epifluorescence microscopy. Part B: Transfected COS-7 cells were incubated with cyproterone acetate, hydroxyflutamide, bicalutamide, and nilutamide (1 μM) for 3 h at 37 °C, and GA (1 μg/mL) was added and incubated for 30 min at 37 °C. Cells were observed and recorded directly by epifluorescence microscopy coupled to a CCD camera.

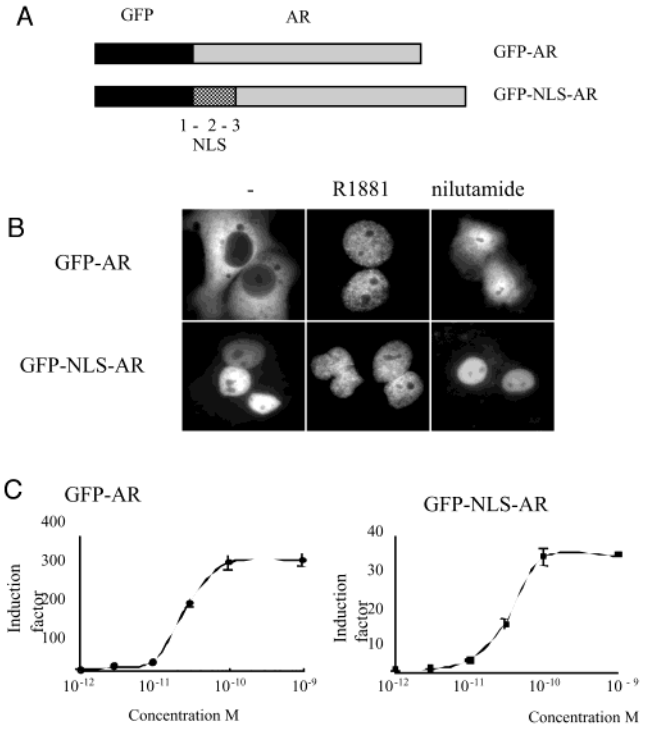

FIGURE 4: Functional characteristics of a constitutively nuclear receptor (GFP-NLS-AR). Part A: Insertion of three copies of the nuclear localization signal (NLS) of SV40 large T antigen between GFP and AR provides a constitutively nuclear receptor, GFP-NLS-AR. Part B: COS-7 cells were transfected with pGFP-AR or pGFP-NLS-AR and observed without hormone or after incubation at 37 °C with R1881 (0.1 μM) or nilutamide (1 μM). Part C: Transcriptional activity of GFP-AR and GFP-NLS-AR was analyzed by reporter gene assays. CV-1 cells were transfected with pGFP-AR or pGFP-NLS-AR and the androgen-dependent reporter gene MMTV-luciferase. Cells were incubated with various concentrations of R1881, and luciferase activities were measured after 30 h.

as the partial antiandrogen cyproterone acetate, presented identical antagonist activities with GFP-NLS-AR and GFP-AR (data not shown). As previously reported (*21*, *22*, *27*), the presence of these nuclear foci formed by GFP-AR and GFP-NLS-AR was correlated to the transcriptional activity.

To further evaluate the role of hsp90 activity in the nucleus, we measured the GA effect on the transcriptional activity of GFP-NLS-AR and GFP-AR. GA's toxic effect on GFP-AR transfected cells was monitored by 30 h of incubation with R1881 with treatment during the last 8 or 10 h with GA. Addition of GA resulted in a slight decrease of 15−20% of total luciferase activity, suggesting that GA does not drastically affect cell viability (Figure 5, panel GA control). On the opposite, simultaneous incubation of R1881 and GA for 10 h at 37 °C resulted in a drastic inhibition of GFP-NLS-AR and GFP-AR transcriptional activities. We then incubated cells at 4 °C with R1881 for 2 h, prior to shifting the temperature to 37 °C with or without GA. In the absence of GA, luciferase activity was slightly decreased compared with the first condition because the incubation at 37 °C was shorter (8 instead of 10 h). As expected, GA decreased GFP-AR transcriptional activity and was related to the reduced nuclear transfer observed in Figure 3A. Figure 5 shows that GA strongly affected the transcriptional activity of androgen-bound GFP-NLS-AR, demonstrating an active role of hsp90, even after nuclear localization. Impairment of AR transcriptional activity was confirmed by the effect of GA on the subnuclear localization of GFP-NLS-AR. In the absence of agonist, addition of GA induced few aggregates in the cytoplasm, corresponding to the 20% remaining cytoplasmic GFP-NLS-AR in the absence of hormone, but the nuclear fluorescence stayed diffuse (Figure 6). When GA was added 3 h after addition of R1881 at 37 °C, foci formation was not inhibited. Conversely, addition of GA after incubation of R1881 at 4 °C inhibited formation of nuclear foci (Figure 6), and the fluorescence stayed homogeneous. These data suggest that hsp90 was required to maintain the aporeceptor in a conformation able not only to bind the ligand but also to acquire an active conformation after agonist binding.

These results demonstrate that GA inhibition of hsp90 activity was able to block both the cytoplasmic GFP-AR and the nuclear GFP-NLS-AR activation. The two fusion proteins had the same sensitivity to GA, and hormone binding at 4 °C in whole cells was not efficient enough to prevent GA action. This experiment suggests that GA blocked a step necessary to acquire a conformation that is able not only to translocate wild-type receptor to the nucleus but also to bind the nuclear matrix and activate transcription.

DISCUSSION

An unresolved question in antiandrogen treatment is how AR antagonists prevent androgen effects. Antiandrogens may act through any step in the cascade of steroid receptor activation, such as ligand binding, nuclear import, DNA binding, or modulation of transcription. Cyproterone acetate is a partial agonist that induces transcriptional activity at high

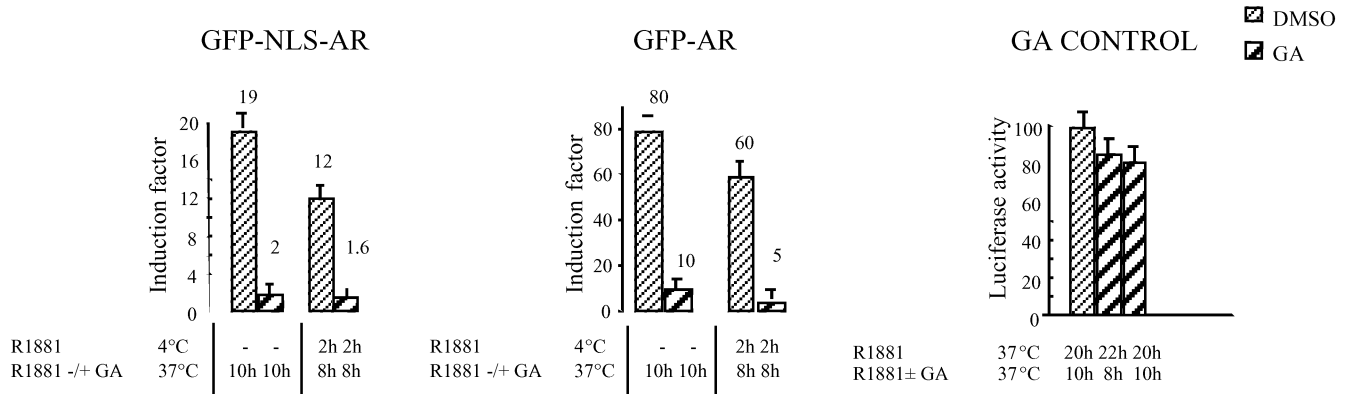

FIGURE 5: GA effects on transcriptional activity of GFP-NLS-AR and GFP-AR. CV-1 cells were transfected with pGFP-NLS-AR or pGFP-AR and the reporter gene MMTV-luciferase. Cells were then incubated with R1881 and GA at 37 °C for 10 h or with R1881 for 2 h on ice prior to addition or not of GA for 8 h at 37 °C. The luciferase activity was then measured on the cell extract and expressed as the induction factor compared to that in the absence of hormone. Control of GA effect on cell viability was performed on CV-1 cells transfected with pGFP-AR and MMTV-luciferase and incubated during 30 h with R1881. GA was added during the last 8 or 10 h of the incubation.

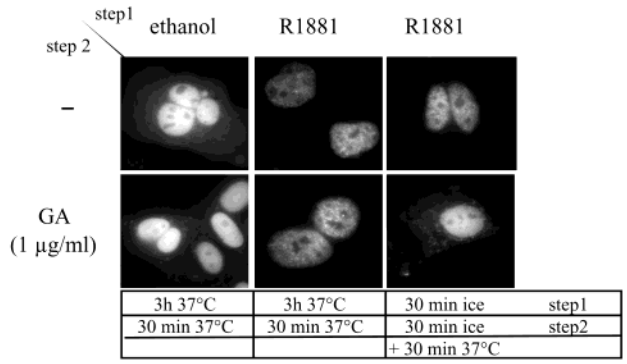

FIGURE 6: Effect of hsp90 inactivation on GFP-NLS-AR subnuclear localization. COS-7 cells expressing GFP-NLS-AR were observed after hormonal treatment by epifluorescence microscopy and recorded with a CCD camera. Incubation of cells with vehicle or R1881 (0.1 μM) was followed by vehicle or GA (1 μg/mL) treatment. The time and temperature used for incubation are indicated.

concentration, and nilutamide and bicalutamide are pure antiandrogens that exhibit only antagonist activity (*23*−*25*). The transcriptional activity triggered by these antihormones was correlated with the equilibrium between the two conformations of AR: an active conformation or DNA-binding form producing a 29 kDa resistant fragment in limited proteolysis assays and an inactive conformation resulting in a 35 kDa resistant band. All tested antiandrogens were able to reduce the androgen-dependent nuclear transfer rate of AR. The partial agonist, cyproterone acetate, slightly slowed the nuclear import caused by androgen while nilutamide and bicalutamide strongly repressed AR nuclear transfer. The transfer rate was correlated with the amount of AR in the active conformation. In the present study, it appears that antiandrogens that reduced the transfer rate into the nucleus are pure antiandrogens and produce a single band of 35 kDa in proteolysis assays.

It has been suggested that the shift from 35 to 29 kDa is correlated with the release of associated proteins from the heterocomplex. Pure antiandrogens would prevent a conformational change by stabilizing a complex with associated proteins (*11*). To explore the role of hsp90 in AR activity, the GA effect was evaluated in both in vitro assays and live cells. The analysis of proteolysis assays in the presence of GA established that GA binding did not protect AR probably by a reduction in the ligand-binding activity of the AR−GA complex (*14*) and slightly affected the conformational change induced by R1881, since R1881 preincubation at 4 °C before addition of GA yielded a lower amount of the 29 kDa band. In the presence of GA, the 35 kDa band observed with cyproterone acetate and nilutamide was proteolyzed, suggesting that the inactive form of AR requires a functional hsp90. The role of the hsp complex in the steroid receptor nuclear import has not yet been well defined. Galigniana et al. reported the inhibition of nuclear transfer of GFP-GR by GA (*16*) and hence demonstrated the hsp90 binding-dependent translocation that validated the transportosome model proposed by Pratt (*26*). More recently, Davies et al. reported that the switch between immunophilins FKBP51 and FKBP52 in the heterocomplexes controls the glucocorticoid receptor subcellular localization and nuclear transport (*27*). In live cells, GA prevented AR migration to the nucleus and created cytoplasmic aggregates. The localization of aggregates observed in the presence of GA would correspond to the sites of proteolytic degradation as proteasome, as previously described after GA treatment for proteins like erb2 (*28*). It appears that hsp90 or associated proteins are involved in the protection of GFP-AR even bound to agonist or antagonist and are required for the nuclear transport of GFP-AR. The heteroprotein complex plays a critical role in maintaining the conformation of nonactivated receptor (no ligand, bound to antiandrogens or agonists at 4 °C).

The nuclear import kinetics of AR bound to antiandrogens reflect an equilibrium between the different heterocomplexes associated with AR, the inactive complex being very sensitive to GA. Nuclear transfer, however, does not seem to be the only step affected by inactive conformation and involved in antagonist activity. In fact, (1) the receptor was nuclear after 24 h of antiandrogen treatment, and (2) the pure antiandrogens conserved their antagonist activity on the constitutively nuclear receptor GFP-NLS-AR. A further step in the action of androgen is the attachment to the nuclear matrix. Tyagi et al. described a punctuate distribution pattern of GFP-AR caused by dihydrotestosterone, while in the case of bicalutamide, the translocated receptor was evenly distributed (*29*). A correlation has been established between these clusters and the transcriptional activity not only for AR (*22*, *29*, *30*) but also for estrogen receptor (*31*). More recently, these

clusters were analyzed by a tridimensional microscopic approach and described as potential sites for formation of precomplex between receptor and coactivators (*30*). We observed the same AR distribution, and here we noted that the addition of GA to GFP-NLS-AR bound to agonist at 4 °C impairs the formation of foci. These data suggest that in the nucleus AR is still associated with hsp90s, which are required to induce an active conformation able to interact with the nuclear matrix. Pure antiandrogens such as nilutamide also impair the formation of active conformation, resulting in several alterations in AR nuclear transfer, association to the nuclear matrix, and interactions with coactivators. The more efficient the antagonist, the more inhibited each step is. The nuclear transfer kinetics are a good indicator of this efficiency, although nuclear transfer is a necessary but not sufficient step for transcription activation. Other antagonists might act on only one step, such as interaction with coactivators, since association with the nuclear matrix was observed with tamoxifen by Stenoien et al. (*31*) but impeded by pure antiestrogens such as ICI182780. Davies et al. observed that RU 486 did not impede the nuclear transfer of GR (*27*).

In conclusion, hsp90 or associated proteins seem to play an important role in nuclear transfer even after agonist binding, since an alteration in this interaction by GA leads to formation of cytoplasmic aggregates. In the nucleus, agonist-bound AR interacts with coactivators and transcriptional factors that may relay the stabilization provided by hsp. In contrast, the inactive conformations induced by nilutamide and bicalutamide are unable to interact with these factors and require interaction with hsp90. Since hsp90 participates in AR stabilization, GA may lead to the rapid downregulation of AR bound to antiandrogen and would thus increase the efficiency of antiandrogens used for prostate cancer treatment.

ACKNOWLEDGMENT

We thank Dr. H. Richard-Foy for the MMTV-luc reporter gene.

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995) *Cell 83*, 835−839.
2. Brinkmann, A. O., Blok, L. J., de Ruiter, P. E., Doesburg, P., Steketee, K., Berrevoets, C. A., and Trapman, J. (1999) *J. Steroid Biochem. Mol. Biol. 69*, 307−313.
3. Cheung, J., and Smith, D. F. (2000) *Mol. Endocrinol. 14*, 939−946.
4. Gehring, U. (1998) *Vitam. Horm. 54*, 167−205.
5. Pratt, W. B., and Toft, D. O. (1997) *Endocr. Rev. 18*, 306−360.
6. Kuil, C. W., and Brinkmann, A. O. (1996) *Eur. Urol. 29* (Suppl. 2), 78−82.
7. Georget, V., Lobaccaro, J., Térouanne, B., Mangeat, P., Nicolas, J., and Sultan, C. (1997) *Mol. Cell. Endocrinol. 129*, 17−26.
8. Georget, V., Terouanne, B., Nicolas, J., and Sultan, C. (1999) in *Methods in Enzymology*, pp 121−135, Academic Press, San Diego, CA.
9. Kuil, C. W., and Mulder, E. (1994) *Mol. Cell. Endocrinol. 102*, R1−R5.
10. Kallio, P. J., Janne, O. A., and Palvimo, J. J. (1994) *Endocrinology 134*, 998−1001.
11. Kuil, C. W., Berrevoets, C. A., and Mulder, E. (1995) *J. Biol. Chem. 270*, 27569−27576.
12. Whitesell, L., and Cook, P. (1996) *Mol. Endocrinol. 10*, 705−712.
13. Czar, M. J., Galigniana, M. D., Silverstein, A. M., and Pratt, W. B. (1997) *Biochemistry 36*, 7776−7785.
14. Segnitz, B., and Gehring, U. (1997) *J. Biol. Chem. 272*, 18694−18701.
15. Prima, V., Depoix, C., Masselot, B., Formstecher, P., and Lefebvre, P. (2000) *J. Steroid Biochem. Mol. Biol. 72*, 1−12.
16. Galigniana, M. D., Scruggs, J. L., Herrington, J., Welsh, M. J., Carter-Su, C., Housley, P. R., and Pratt, W. B. (1998) *Mol. Endocrinol. 12*, 1903−1913.
17. Brown, T. R., Lubahn, D. B., Wilson, E. M., French, F. S., Migeon, C. J., and Corden, J. L. (1990) *Mol. Endocrinol. 4*, 1759−1772.
18. Gouilleux, F., Sola, B., Couette, B., and Richard-Foy, H. (1991) *Nucleic Acids Res. 19*, 1563−1569.
19. Pines, J. (1995) *Trends Genet. 11*, 326−327.
20. Georget, V., Terouanne, B., Lumbroso, S., Nicolas, J., and Sultan, C. (1998) *J. Clin. Endocrinol. Metab. 83*, 3597−3603.
21. Avances, C., Georget, V., Terouanne, B., Orio, F., Cussenot, O., Mottet, N., Costa, P., and Sultan, C. (2001) *Mol. Cell. Endocrinol. 184*, 13−24.
22. Tomura, A., Goto, K., Morinaga, H., Nomura, M., Okabe, T., Yanase, T., Takayanagi, R., and Nawata, H. (2001) *J. Biol. Chem. 276*, 28395−28401.
23. Terouanne, B., Tahiri, B., Georget, V., Belon, C., Poujol, N., Avances, C., Orio, F., Jr., Balaguer, P., and Sultan, C. (2000) *Mol. Cell. Endocrinol. 160*, 39−49.
24. Kemppainen, J. A., Lane, M. V., Sar, M., and Wilson, E. M. (1992) *J. Biol. Chem. 267*, 968−974.
25. Kemppainen, J. A., Langley, E., Wong, C. I., Bobseine, K., Kelce, W. R., and Wilson, E. M. (1999) *Mol. Endocrinol. 13*, 440−454.
26. Pratt, W. B. (1993) *J. Biol. Chem. 268*, 21455−21458.
27. Davies, T. H., Ning, Y. M., and Sanchez, E. R. (2002) *J. Biol. Chem. 277*, 4597−4600.
28. Tikhomirov, O., and Carpenter, G. (2000) *J. Biol. Chem. 275*, 26625−26631.
29. Tyagi, R. K., Lavrovsky, Y., Ahn, S. C., Song, C. S., Chatterjee, B., and Roy, A. K. (2000) *Mol. Endocrinol. 14*, 1162−1174.
30. Saitoh, M., Takayanagi, R., Goto, K., Fukamizu, A., Tomura, A., Yanase, T., and Nawata, H. (2002) *Mol. Endocrinol. 16*, 694−706.
31. Stenoien, D. L., Mancini, M. G., Patel, K., Allegretto, E. A., Smith, C. L., and Mancini, M. A. (2000) *Mol. Endocrinol. 14*, 518−534.

BI0259150