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Endogenous Coactivator ARA70 Interacts with Estrogen Receptor α (ERα) and Modulates the Functional ERα/Androgen Receptor Interplay in MCF-7 Cells*

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Overexpression of androgen receptor (AR) decreases estrogen receptor α (ERα) transactivation, which plays a basic role in hormone-dependent breast cancer. This transcriptional interference can be due to shared coactivators. Here we demonstrated that in MCF-7 cells ARA70, an AR-specific coactivator, interacted with endogenous ERα, increasing its transcriptional activity, and it was recruited to the *pS2* gene promoter. Moreover, a dominant negative ARA70 down-regulated ERα transcriptional activity as well as *pS2* mRNA. ARA70 overexpression reversed the AR down-regulatory effect on ERα signaling. However, in the presence of a progressive increase of transfected AR, ARA70 switched into enhancing the inhibitory effect of AR on ERα signaling. These opposite effects of ARA70 were further evidenced by coimmunoprecipitation assay in MCF-7wt, MCF-7-overexpressing AR, and HeLa cells, exogenously expressing an excess of ERα with respect to AR or an excess of AR with respect to ERα. Thus, ARA70 is a coactivator for ERα and may represent a functional link between ERα/AR modulating their cross-talk in models of estrogen signaling in MCF-7 and HeLa cells.

In the last decade, the molecular mechanism involved in breast cancer hormone dependence has been extensively investigated. The growth of breast cancer cells is responsive to various growth factors and steroid hormones and also reflects the cross-talk between the respective signaling pathways. The role of nuclear receptors in breast cancer is an intriguing area of investigation, which continues to broaden as the number of coactivators and corepressors influencing the link between steroid receptors and the cell transcriptional machinery has grown.

The prognostic impact of estrogen receptor and progesterone receptor (PR)[1] in breast cancer is well established because they both do predict a good response rate of breast cancer to hormonal treatment (1, 2). By contrast, even though the androgen receptor is present in 70–90% of primary breast tumors, and it is often coexpressed with the ERα and PR in human breast tumors, its clinical significance and functional role in breast cancer is poorly understood (3). ARs are expressed in normal mammary gland, which is also an androgen target tissue. Androgens often antagonize the effects of estrogens in mammary gland development and could be considered to be endogenous anti-estrogens under some circumstances (4).

Several lines of evidence support the idea that this interplay is biologically meaningful. 1) Reduced or impaired AR signaling has been implicated in the development of hereditary male breast cancers (5). 2) Women who have AR alleles that encode for short polyglutamine tracts in the AR protein have a reduced risk of developing breast cancer (6). These observations suggest that androgen signaling in the breast might protect against cancer development and progression. On the basis of such findings, androgens have been used successfully to treat metastatic female breast cancers with comparable efficacy to tamoxifen, but the treatment was not well tolerated because of its masculinizing side effects (7).

Because androgens have been shown to inhibit the proliferation of AR-positive breast cancer cell lines in culture (8), in our previous studies we attempted to evaluate the role of the AR in the control of the proliferation of the hormone-dependent breast cancer cell line MCF-7. These experiments demonstrated that the potent androgen dihydrotestosterone (DHT) inhibits both basal and estrogen-induced cell proliferation. This effect of DHT on MCF-7 cell proliferation was reversed by the androgen receptor antagonist, hydroxyflutamide (OHFl), indicating that it was mediated via an interaction with the AR. Moreover overexpression of the AR in MCF-7 cells markedly decreased ERα transcriptional activity, suggesting a functional cross-talk between the two transduction pathways (9).

Transcriptional interference or "squelching" between various steroid receptors has been interpreted as a reflection of the involvement of shared coactivator proteins that mediated functional interactions between nuclear receptors, the basal transcriptional machinery, and the chromatin environment (10, 11). Therefore, the ability of overexpressed AR to inhibit ERα-dependent signaling in MCF-7 suggests that these transcription factors utilize a common pool of coactivators that are

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[1] The abbreviations used are: PR, progesterone receptor; AR, androgen receptor; ERα, estrogen receptor α; DHT, dihydrotestosterone; OHFl, hydroxyflutamide; dARA70fl, dominant negative full-length ARA70; DBD, DNA-binding domain; LBD, ligand-binding domain; ChIP, chromatin immunoprecipitation; ERE, estrogen-responsive element; DMEM, Dulbecco's modified Eagle's medium; E_2, estradiol.

present in limiting cellular concentration. Here we evaluated the role of ARA70, previously identified as an AR specific coactivator (12), in the modulation of ERα signal by the AR.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM/F-12), L-glutamine, Eagle's non-essential amino acids, penicillin, streptomycin, calf serum, bovine serum albumin, and phosphate-buffered saline were purchased from Eurobio (Les Ullis Cedex, France). Triazol reagent was obtained from Invitrogen, and FuGENE 6 was from Roche Applied Science. TaqDNA polymerase, 100-bp DNA ladder, dual luciferase kit, and thymidine kinase *Renilla* luciferase plasmid were provided by Promega (Madison, WI). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and sodium orthovanadate were purchased from Sigma. Antibodies against ERα, AR, ARA70, and β-actin and salmon sperm DNA/protein A-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated horse anti-goat IgG and ABC complex/horseradish peroxidase were provided by Vector Laboratories. Chromogen, 3-diaminobenzidine tetrachloride dihydrate, was purchased from Bio-Optica. ECL System was purchased from Amersham Biosciences. VCX500 ultrasonic processor was provided by Sonics (Newtown, CT).

Plasmids—Firefly luciferase reporter plasmid XETL is a construct containing an estrogen-responsive element from the *Xenopus* vitellogenin promoter (13). The wild type human ERα expression vector (HEGO) consists of the full-length ERα cDNA fused with the SV40 early promoter and expressed in the pSG5 vector (14). The full-length androgen receptor expression plasmid is described by Tilley *et al.* (15). The expression plasmid containing the a human AR complementary DNA carrying a mutation in the DNA binding domain (Cys-574 → Arg) is cytomegalovirus P881 (16). The expression plasmid containing a cDNA encoding a constitutively active AR deleted for the ligand binding domain (LBD) (AR-(1–707)) was described previously (17). pSG5-ARA70 plasmid encoding the full-length ARA70 (12) and dominant negative mutant ARA70 pSG5-dARA70fl (18) was a gift from Dr. C. Chang, Rochester Medical Center, Rochester, NY. The *Renilla* luciferase expression vector pRL-CMV (Promega) was used as a transfection standard.

Cell Culture—Wild type human breast cancer MCF-7 cells were gifts from Dr. B. Van der Burg (Utrecht, The Netherlands). Human uterine cervix adenocarcinoma (HeLa) cells were obtained from the ATCC (Manassas, VA). MCF-7 and HeLa cells were maintained in DMEM/F-12 medium containing 5% fetal calf serum, 1% L-glutamine, 1% Eagle's nonessential amino acids, and 1 mg/ml penicillin/streptomycin in a 5% CO_2 humidified atmosphere. Cells were cultured in phenol red-free DMEM, 0.5% bovine serum albumin, and 2 mM L-glutamine (serum-free medium), for 24 h before each experiment. Hormone stimulation was performed in DMEM containing 5% charcoal-treated fetal calf serum to reduce the endogenous steroid concentration (19).

Immunocytochemical Staining—Paraformaldehyde-fixed MCF-7 cells (2% paraformaldehyde for 30 min) were used for immunocytochemical staining. Endogenous peroxidase activity was inhibited by pretreatment with hydrogen peroxide (3% in absolute methanol for 30 min), and nonspecific sites were blocked by incubation with normal horse serum (10% for 30 min). ARA70 immunostaining was then performed using as primary antibody a goat polyclonal antiserum (1:40, overnight at 4 °C), whereas a biotinylated horse anti-goat IgG (1–600, for 1 h at room temperature) was utilized as secondary antibody. Avidin-biotin-horseradish peroxidase complex (ABC complex/horseradish peroxidase complex) was applied (30 min), and the chromogen 3–3′-diaminobenzidine tetrachloride dihydrate was used as the detection system (5 min). TBS-T (0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6, containing 0.05% Triton X-100) served as washing buffer. The primary antibody was replaced by normal goat serum at the same concentration in control experiments on MCF-7 cultured cells.

Reverse Transcription-PCR Assay—Total cellular RNA was extracted from MCF-7 cells using Triazol reagent as suggested by the manufacturer. 2 μg of total RNA were reverse-transcribed using 200 IU of Moloney murine leukemia virus-reverse transcriptase in a reaction volume of 20 μl (0.4 μg of oligo(dT), 0.5 mM deoxy-NTP, and 24 IU of RNasin) for 30 min at 37 °C, followed by heat denaturation for 5 min at 95 °C.

2 μl of cDNA were then amplified with a pair of 5′ and 3′ 50-pmol primers for either ARA70, *pS2*, or 36B4 by using 2 units of TaqDNA polymerase. The primers used are as follows: ARA70: forward, 5′-AAGACCAGAGTGGCAGCTCCAGTAAT-3′, and reverse, 5′-CTAGCATGAGCCATCAAGTGCTCA-3′, to amplify a 520-bp fragment; *pS2*: forward, 5′-TTCTATCC-TAATACCATCGACG-3′, and reverse, 5′-TTTGAGTAGTCAAAGTCAGAGC-3′, to amplify a 210-bp fragment; ribosomal RNA 36B4: forward, 5′-CTCAACATCTCCCCCTTCTC-3′, and reverse, 5′-CAAATCCCATATCCTCGTCC-3′, to amplify a 408-bp fragment. PCR amplification was performed in 10 mM Tris-HCl, pH 9.0, containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.25 mM each dNTP. The cycling conditions for ARA70 were 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The cycling conditions for *pS2* and 36B4 were 95 °C for 5 min followed by 20 cycles of 94 °C for 1 min, 59 °C for 2 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Equal amounts of PCR product were electrophoresed on a 1% agarose gels and visualized by ethidium bromide staining. To check out for the presence of DNA contamination, a reverse transcription-PCR was performed without Moloney murine leukemia virus-reverse transcriptase (negative control).

Western Blotting and Immunoprecipitation Analysis—Whole cell extracts were prepared by lysing cells in buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 3 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate) and centrifugation to remove the insoluble material. For Western blotting assay, 50 μg of the protein lysates were separated on SDS-10% polyacrylamide gels and electroblotted to nitrocellulose membrane. Membranes were incubated 1 h with 5% non fat dry milk in 0.2% Tween 20 in Tris-buffered saline (TBS-T) at room temperature and then probed with an appropriate dilution of the various antibodies as indicated in the figure legends, followed by anti-goat or anti-mouse horseradish peroxidase-conjugated antibody. Blots were developed using the ECL Plus Western blotting detection system according to the manufacturer's instructions. For immunoprecipitation assay, 500 μg of the protein lysates were incubated for 2 h with protein A/G-agarose beads at 4 °C and centrifuged at 12,000 × *g* for 5 min, to avoid nonspecific binding. The supernatants were incubated overnight with 10 μl of anti-ARA70 and 500 μl of HNTG (IP) buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin) for each. Immune complexes were recovered by incubation with protein A/G-agarose. The beads containing bound proteins were washed three times by centrifugation in IP buffer, then denatured by boiling in Laemmli sample buffer, and analyzed by Western blot to identify the coprecipitating effector proteins. Immunoprecipitation with protein A/G alone was used as the negative control. Membranes were stripped of bound antibodies by incubation in glycine 0.2 M, pH 2.6, for 30 min at room temperature. Prior to reprobing with different primary antibodies, stripped membrane were washed extensively in TBS-T and placed in blocking buffer (TBS-T containing 5% milk) overnight.

Transfection Assay—MCF-7 and HeLa cells were grown into 24-well plates. The medium was replaced with DMEM lacking phenol red as well as serum on the day of transfection, which was performed using the FuGENE 6 Reagent as recommended by the manufacturer. All transfection mixture contained 0.5 μg of reporter plasmid XETL. XETL was cotransfected alone or in combination with ERα and/or AR full-length, AR (Cys-574 → Arg), AR-(1–707), ARA70 expression plasmids. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. In addition, to assess transfection efficiency, each DNA mixture contained 5 ng of pRL-TK-Luc, a plasmid encoding *Renilla* luciferase. Upon transfection, the cells were shifted to serum-free medium for 6 h and then treated with 10^{-7} M E_2 or 10^{-7} M DHT or left untreated for 18 h. Luciferase activity in cell lysates was measured using the dual luciferase assay system following the manufacturer's instructions. The firefly luciferase data for each sample were normalized on the basis of transfection efficiency measured by *Renilla* luciferase activity.

For whole cell extracts, cells were plated on a 10-cm dish and transfected, using the FuGENE 6 reagent, with an appropriate amount of the various plasmids as indicated in the figure legends. Upon transfection cells were shifted to serum-free medium for 6 h and then treated with 10^{-7} M E_2 or 10^{-7} M DHT or left untreated in DMEM containing 5% charcoal-treated fetal calf serum for 48 h.

For chromatin immunoprecipitation (ChIP) assay samples, cells were grown in 150-mm plates and transfected, using the FuGENE 6 reagent, with an appropriate amount of the various plasmids as indicated in the figure legends.

ChIP and Re-ChIP Assay—We followed the ChIP methodology described by Shang *et al.* (20) with minor modifications. MCF-7 cells were grown in 100-mm plates. 90% confluent cultures were shifted to serum-free medium for 24 h and then treated with 10^{-7} M E_2 for 45 min or were left untreated in serum-free medium. Following treatment, the cells were washed twice with phosphate-buffered saline and cross-linked with 1% formaldehyde at 37 °C for 10 min. Next the cells were washed

twice with phosphate-buffered saline at 4 °C, collected, and resuspended in 200 μl of Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1) and left on ice for 10 min. Then the cells were sonicated four times for 10 s at 30% of maximal power and collected by centrifugation at 4 °C for 10 min at 14,000 rpm. Supernatants were collected and diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 μl of sonicated salmon sperm DNA/protein A-agarose for 1 h at 4 °C. The precleared chromatin was immunoprecipitated for 12 h either with anti-ARA70 goat antibody or with normal goat IgG (Santa Cruz Biotechnology) as the negative control. After that, 60 μl of salmon sperm DNA/protein A-agarose was added, and precipitation was continued for 2 h at 4 °C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immune complexes were eluted with Elution buffer (1% SDS, 0.1 M $NaHCO_3$). The eluates were reverse cross-linked by heating at 65 °C for 12 h and digested with 0.5 mg/ml proteinase K at 45 °C for 1 h. DNA was obtained by phenol and phenol/chloroform extractions. 2 μl of 10 mg/ml yeast tRNA were added to each sample, and DNA was precipitated with ethanol for 12 h at −20 °C and resuspended in 20 μl of TE buffer. 5 μl of each sample were used for PCR with the following *pS2* promoter primers: upstream 5′-GATTACAGCGTGAGCCACTG-3′ and downstream 5′-TGGTCAAGCTACATGGAAGC-3′. The PCR conditions were as follows: 45 s at 94 °C, 45 s at 58 °C, and 90 s at 72 °C. The amplification products, obtained in 35 cycles, were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. In Re-ChIP experiments, complexes were eluted by incubation for 30 min in Re-IP buffer (0.5 mM dithiothreitol, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl) and subjected again to the ChIP procedure, using anti-ERα antibody (F10, Santa Cruz Biotechnology) and normal mouse antibody (Santa Cruz Biotechnology) for the negative control.

Statistical Analysis—Each datum point represents the mean ± S.E. of three different experiments. Data were analyzed by analysis of variance test using the STATPAC computer program.

RESULTS

Prolonged Exposure to DHT Increases AR Content and Decreases ERα Expression in MCF-7 Cells—We demonstrated previously (9) that DHT inhibits both basal and estrogen-induced cell proliferation of a hormone-dependent breast cancer cell line MCF-7 (9).

The effect of DHT addition on AR and ERα content in MCF-7 cells was evaluated by a time course study (Fig. 1). An increase of endogenous AR protein expression, and a decrease in ERα content, can be observed after 6 h of androgen exposure, and it lasts until the 6th day of treatment. Both changes reflect alterations that would be expected following stimulation with the respective ligand (*e.g.* AR with DHT and ERα with estradiol). However, the use of DHT, which cannot be converted into estrogens by aromatase, suggests that the down-regulation of ERα, observed in such circumstances, is because of activation of the AR with its own ligand.

ERα Transcriptional Activity Is Inhibited by Overexpressed AR—We investigated whether the down-regulatory effect of AR on estrogen signaling may be related not only to the induced reduction of the ERα content but also to a direct interference on ERα transcriptional machinery.

The ability of AR to modulate ERα transcriptional activity was examined in MCF-7 cells, expressing endogenous ERα and AR, and HeLa cells, ectopically expressing the two steroid receptor proteins (Fig. 2). A firefly luciferase reporter plasmid (XETL), containing an estrogen-responsive element (ERE) sequence upstream of a thymidine kinase promoter, was transiently transfected in MCF-7 (Fig. 2*B*) and HeLa cells expressing ectopic ERα (Fig. 2*C*) in the presence of an expression plasmid encoding the full-length AR. After transfection, cells were either left untreated or treated with 10^{-7}

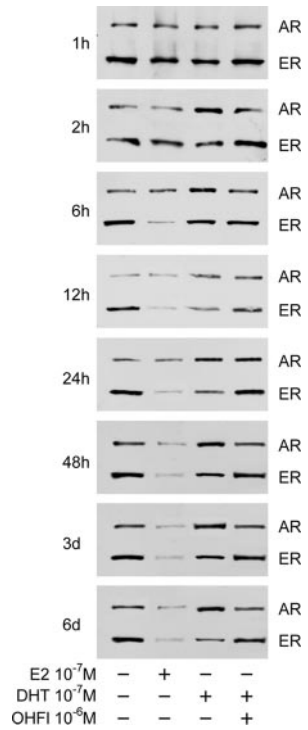

FIG. 1. **Effect of prolonged exposure to DHT on AR and ERα content in MCF-7 cells.** MCF-7 cells were grown in the absence or presence of steroids as indicated. Whole cell extracts were prepared and subjected to immunoblotting with mouse monoclonal anti-AR antibody. Nitrocellulose filter was then stripped and assayed to determine the level of immunoreactive ERα using a mouse monoclonal anti-ERα antibody.

M E_2. After 18 h, cells were harvested, and luciferase activity was determined.

As shown in Fig. 2*B*, in MCF-7 cells the overexpression of AR decreased the E_2-induced signal by about 60% when compared with hormone stimulated activity in the absence of exogenous AR. This effect was partially reversed by the addition of OHFl (data not shown). Similar results were obtained in HeLa cells (Fig. 2*C*).

To examine whether the effect of transfected AR on ERα-dependent transcription is because of its transactivation properties, luciferase gene expression was determined in MCF-7 and HeLa cells (expressing ectopic ERα) cotransfected with an expression plasmid encoding an AR carrying a mutation ($AR_{Cys\text{-}574 \rightarrow Arg}$) in the DNA binding domain (DBD), which disrupts the ability of the AR to bind to target DNA sequences. In these circumstances, there was no significant decrease in luciferase activity.

By contrast, the inhibitory effect on the E_2-induced signal is evident in cells transfected with an expression plasmid encoding a constitutively active mutant AR in which the LBD has been deleted (AR-(1–707)) (Fig. 2, *B* and *C*). As expected, the addition of androgen receptor antagonist OHFl had no effect on the activity of the AR-(1–707) (data not shown).

MCF-7 Human Breast Cancer Cell Line Expresses the ARA70 Protein—The negative interference of AR on ERα transcriptional activity led us to explore whether shared cofactors might be involved in such inhibitory effect. In this concern, we investigated the role of the ARA70 reported to be decreased in breast and prostate cancer (21). Immunohistochemical analysis was performed to examine the localization of the endogenous ARA70 protein in MCF-7 cells. Representative results of immunostaining experiments utilizing a polyclonal antibody to ARA70 are shown in Fig. 3*A*. In MCF-7 cells, immunoreactivity was observed in both the cytoplasmic and nuclear compart-

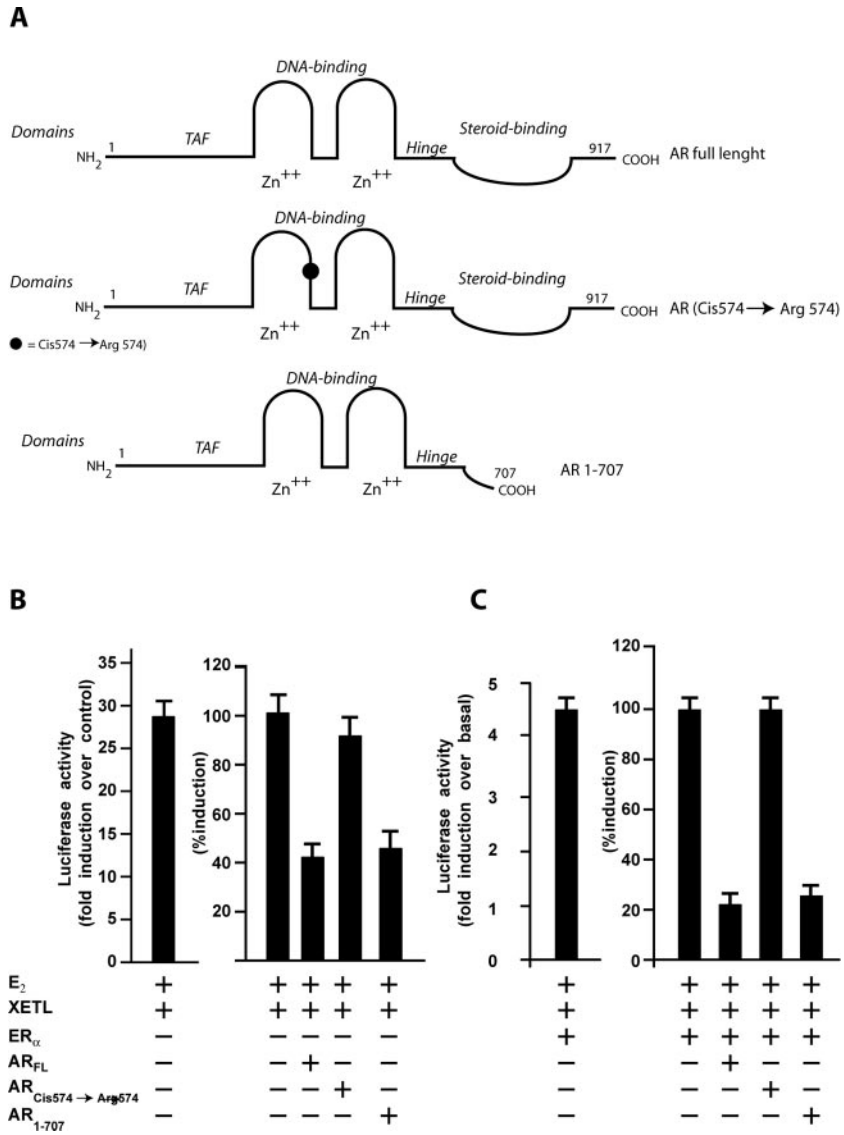

FIG. 2. **AR overexpression inhibits ERα transcriptional activity in MCF-7 cells.** *A*, schematic representation of wild type and mutants of AR utilized in the above described experiments. MCF-7 cells (*B*) and HeLa cells, expressing ectopic ERα (0.5 μg/well) (*C*), were transiently cotransfected with XETL (0.5 μg/well) either in the presence of wild type AR (0.5 μg/well) or one of the mutants of AR (0.5 μg/well) as indicated. After transfection cells were left untreated or treated with 10^{-7} M E_2 and harvested after 18 h, and luciferase activities were determined. In the absence of E_2, no significant level of XETL luciferase activation was observed. Activation of reporter gene expression XETL in the presence of E_2 is arbitrarily set at 100%. Results represent the means ± S.E. of three separate experiments each in duplicate.

ments. No change in the intracellular distribution of ARA70 expression was observed at 24 h following treatment with 10^{-7} M DHT. Immunostaining was not observed in the cells processed without primary antibody. The expression of ARA70 in MCF-7 cells was confirmed by reverse transcription-PCR using a combination of primers amplifying 520 bp of the N-terminal region of ARA70. The expected reverse transcription-PCR product was clearly detected as shown in Fig. 3*B*.

Finally, we performed Western blotting analysis using an antibody against ARA70 protein. As shown in Fig. 3*C*, an immunoreactive 70-kDa band was detected either in absence or presence of 10^{-7} M E_2 or DHT treatment.

ARA70 Physically Interacts with ERα—To assess the possibility that endogenous ARA70 can interact with ERα in MCF-7 intact cells, coimmunoprecipitation assay was carried out. Protein complexes associated with ARA70 were first immunoprecipitated with an anti-ARA70 antibody, and the precipitated proteins were then subjected to immunoblotting with anti-ERα antibody. As shown in Fig. 4*A*, a protein of 67 kDa corresponding to ERα was recognized both in the absence and presence of E_2, with a stronger coprecipitation under treatment, demonstrating an interaction between endogenous ARA70 and ERα. Similar results were obtained when the coimmunoprecipitation assay was performed using an ERα antibody followed by Western blotting with ARA70 antibody (data not shown).

This ERα/ARA70 association appears not to be specifically restricted to the breast cancer cells MCF-7 because similar results were obtained in HeLa cells transfected with an ARA70 expression plasmid in the absence or presence of ERα-expressing vector. A polyclonal anti-ARA70 antibody was employed for coimmunoprecipitation, and the resulting immune complexes were subjected to Western blotting analysis with ERα antibody. A 67-kDa protein corresponding to ERα was detected in immunoprecipitates from cells transfected with both ARA70 and ERα but not from cells expressing ARA70 in the absence of ERα.

ARA70 Stimulates ERα-mediated Transcription—Because the coimmunoprecipitation assay indicated that ARA70 can interact with ERα in an E_2-dependent manner, we were interested in determining whether such an interaction can affect ERα transcriptional activity. To test the possible role of ARA70 in modulating ERα-dependent transactivation, we performed transient transfection experiments in MCF-7 cells and HeLa cells expressing ectopic ERα. An estrogen-dependent reporter construct (XETL) was transfected into MCF-7 cells either in the absence or presence of an expression vector encoding ARA70. After transfection, cells were either left untreated or treated with 10^{-7} M E_2. After 18 h, cells were harvested, and luciferase activity was determined.

The result of such an experiment is shown in Fig. 4*B*. Under our experimental conditions in the absence of E_2, the basal

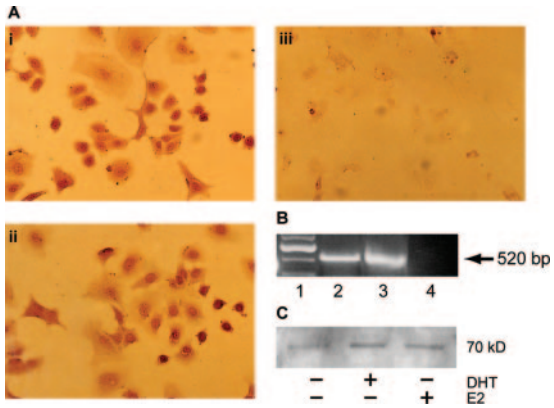

FIG. 3. **MCF-7 cells express ARA70.** *A*, MCF-7 cells were incubated in the absence (*panel i*) or presence of 10^{-7} M DHT (*panel ii*) for 24 h and then fixed and analyzed by staining with human ARA70 antibody. MCF-7 cells incubated without the primary antibody were used as the negative control (*panel iii*). The pictures shown are representative examples of experiments that were performed at least three times with the same results. *B*, total RNA from MCF-7 cells was isolated and reverse-transcribed. The first strand cDNA was subsequently subjected to PCR using ARA70-specific primers. *Lane 1*, 100-bp molecular weight; *lane 2*, MCF-7 cDNA; *lane 3*, ARA70 expression plasmid used as positive control; *lane 4*, MCF-7 RNA sample without the addition of reverse transcriptase (negative control). *Arrow* indicates the position of the 520-bp ARA70 PCR product. *C*, MCF-7 cells were grown in absence (−) or presence (+) of 10^{-7} M E_2 or DHT for 48 h. Whole cell extracts were prepared and subjected to immunoblotting with goat anti-ARA70 antibody.

level of luciferase activity did not change, whereas in the presence of E_2, XETL expression was increased by about 13 ± 0.9-fold. Ectopic expression of ARA70 increased E_2-stimulated ERE-luciferase gene expression by ~80% when compared with the hormone stimulated activity in the absence of exogenous ARA70. No increase in ERα transcriptional activity was observed when exogenous ARA70 was expressed in the absence of exogenous ligand. A similar pattern of results, but with a lesser level of stimulation by E_2, was obtained when ERα and ARA70 expression plasmids were cotransfected in HeLa cells. These patterns are similar whether expressed in terms of absolute level of stimulated reporter gene activity or as fold induction of reporter gene activity.

ERα-ARA70 Complex Binds the Promoter Region of the Estrogen Target Gene pS2 in MCF-7 Cells—After showing that ARA70 is able to modulate ERα transcriptional activity, to explore further whether the ERα-ARA70 interaction may be important *in vivo*, we next investigated the recruitment of ERα-ARA70 complex to the promoter of the well characterized estrogen-responsive target gene *pS2*, either in presence or absence of E_2. MCF-7 cells were grown in the absence of estrogen for 24 h followed by either no treatment or treatment with saturating levels of E_2 for 45 min. The status of the endogenous transcription complex present on the estrogen-responsive region of the *pS2* promoter was determined by using ChIP assay.

The presence of the specific promoter in the chromatin immunoprecipitates, obtained using an anti-ARA70 antibody, was analyzed by semi-quantitative PCR using specific pairs of primers spanning the estrogen-responsive region of *pS2* promoter. As shown in Fig. 5, treatment with E_2 induced an increase in the occupancy by ARA70 of the *pS2* promoter.

On the other hand, subsequent Re-ChIP of the eluates deriving from the primary anti-ARA70 immunoprecipitation, using an anti-ERα antibody, was able to indicate the colocalization of the ERα and ARA70 on the same region of the *pS2* promoter. In other words, these experiments address how ERα and ARA70 act in a combinatorial fashion on the same ERα-responsive promoter.

The Dominant Negative ARA70 Inhibits ERα Transactivation as Well as pS2 Expression in MCF-7 Cells—To investigate further whether interruption or modulation of the ERα-ARA70 interaction could lead to down-regulation of ERα functional activity in response to estradiol, we used a dominant negative approach.

MCF-7 cells that express endogenous ARA70 were transiently transfected with XETL reporter construct in the absence or presence of increasing amounts of full-length dominant negative ARA70 (dARA70fl), which is able to form a nonfunctional heteromer with ARA70 interrupting nuclear receptor transcriptional activity as described previously (18). After 24 h of ligand treatment, cells were harvested, and ERα transcriptional activity was measured as mentioned above. As depicted in Fig. 6*A*, 10^{-7} M E_2 treatment stimulated XETL activity about 13.5-fold compared with untreated cells, whereas in the same experimental conditions dARA70fl transfection inhibited ERα transcriptional activity in a dose-dependent manner.

To determine further the inhibitory effect of dARA70fl on the endogenous ERα target gene, we analyzed the expression of the *pS2* gene widely studied as an estrogen-responsive gene in the breast cancer cell line MCF-7. MCF-7wt or transfected with dARA70fl were treated for 24 h with E_2, as indicated, and then harvested and assayed for *pS2* expression using reverse transcription-PCR analysis. As shown in Fig. 6*B*, in MCF-7wt E_2 treatment induced *pS2* mRNA expression that was significantly inhibited by transfection of a different amount of dARA70fl. These results suggest that dARA70fl functionally inactivates ARA70 and thereby inhibits ARA70-enhanced ERα transactivation, and then down-regulates the expression of the classical E_2-dependent gene, *pS2*, in MCF-7 cells, indicating that ARA70 is involved in ERα transcriptional activity.

Overexpression of ARA70 Reverses AR-induced Inhibition of ERα Transcriptional Activity—To examine directly whether ARA70 could be involved in the transcriptional interference of AR on ERα-induced signals, we tested the effect of ARA70 overexpression on AR-induced inhibition of ERα activity. To achieve such a goal, MCF-7 cells were transiently cotransfected with the XETL reporter construct, either in absence or presence of an AR expression vector and increasing amounts of a plasmid encoding ARA70. After transfection, cells were either left untreated or treated with 10^{-7} M E_2. After 18 h cells were harvested, and luciferase activity was determined. As shown in Fig. 7*A*, in the absence of exogenous AR expression, E_2 treatment increased XETL luciferase activity by ~14-fold. When exogenous AR was simultaneously expressed, most of the activation by E_2 was lost (as described previously). However, when the same experiment was repeated in the presence of a progressive increase in the amount of ectopic ARA70, XETL luciferase activity was restored. Thus, ARA70 overexpression is able to abrogate completely the inhibitory effect induced by the overexpressed AR in MCF-7 cells.

To confirm these findings, the same experiment was carried out in HeLa cells expressing ectopic ERα. As shown in Fig. 7*B*, AR coexpression repressed the E_2-induced luciferase activity by 40%. In the presence of a progressive increase in the amount of ARA70, this repression was not observed. In addition, HeLa cells expressing increasing amounts of ectopic ERα or AR were cotransfected with the XETL plasmid in the absence or presence of an ARA70 expression plasmid.

It is worth noting that in HeLa cells the ectopic overexpression of ARA70 appeared to reduce the amount of ERα required to restore E_2-induced signals (Fig. 8*A*). On the other hand, in the presence of a progressive increase of ectopically expressed AR, ARA70 failed to restore E_2-induced luciferase activity and

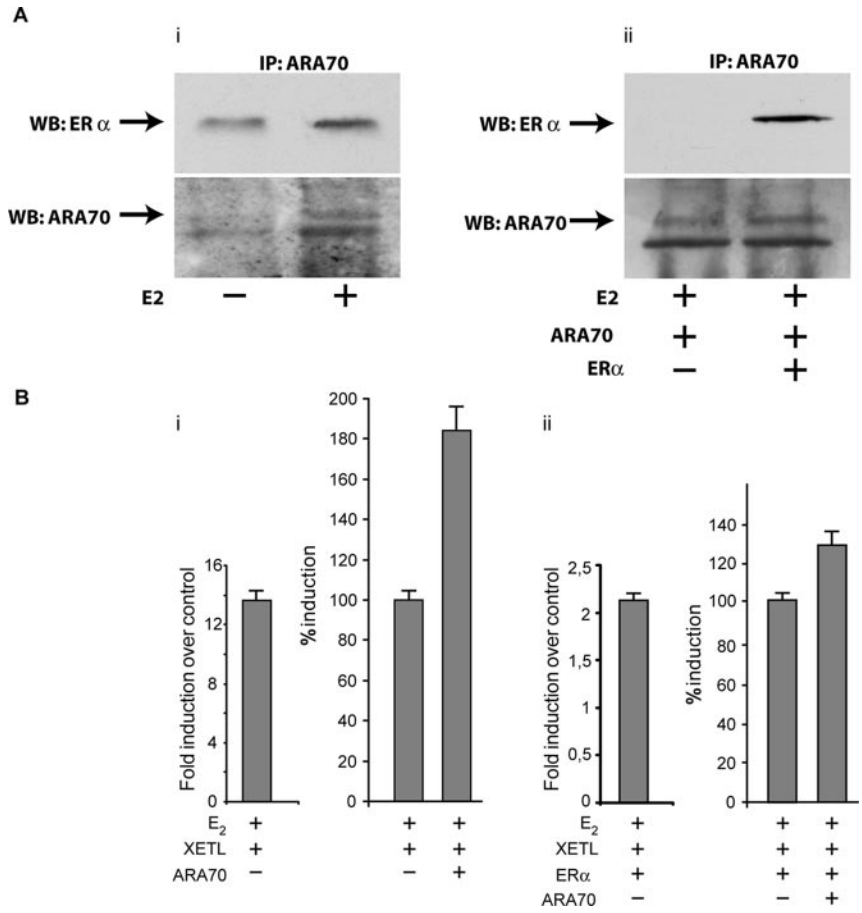

FIG. 4. **ARA70 interacts with ERα and enhances its transcriptional activity in MCF-7 cells and in transfected HeLa cells.** *A*, whole cells extracts were prepared from MCF-7 cells (*panel i*) grown in the absence (−) or presence (+) of 10^{-7} M E_2 for 48 h, and from HeLa cells (*panel ii*) transfected with ARA70 and/or ERα expression plasmid (10 μg/well), as indicated, and grown in the presence of 10^{-7} M E_2 for 48 h. Immunoprecipitation assay was performed using goat anti-ARA70 antibody. Immunoprecipitated (*IP*) proteins were resolved by SDS-PAGE and subjected to immunoblotting with mouse anti-ERα antibody. Nitrocellulose filter was then stripped and blotted with anti-ARA70 antibody. *WB*, Western blot. *B*, MCF-7 cells (*panel i*) were transiently cotransfected with XETL reporter plasmid (0.5 μg/well) either in the absence or presence of full-length ARA70 expression plasmid (2 μg/well). HeLa cells (*panel ii*), expressing ectopic ERα (0.1 μg/well), were transiently cotransfected with XETL reporter plasmid (0.5 μg/well) either in the absence or presence of full-length ARA70 expression plasmid (0.5 μg/well). After transfection cells were left untreated or treated with 10^{-7} M E_2 and harvested after 18 h, and luciferase activities were determined. In absence of E_2, no significant level of XETL luciferase activation was observed. Activation of reporter gene expression XETL in the presence of E_2 is arbitrarily set at 100%. Results represent the means ± S.E. of three separate experiments each in duplicate.

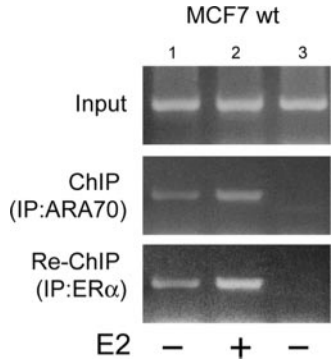

FIG. 5. **Recruitment of ARA70 to the ERE-containing *pS2* promoter in MCF-7 cells.** ChIP assay: soluble, precleared chromatin was obtained from MCF-7wt cells treated with 10^{-7} M E_2 for 1 h (+) or left untreated (−), and immunoprecipitated (*IP*) with anti-ARA70 antibody or with normal goat IgG as negative control (*lane 3*). *pS2* promoter sequences containing ERE were detected by PCR amplification with specific primers. To determine input DNA, the *pS2* promoter fragment was amplified from 5 μl of purified soluble chromatin before immunoprecipitation. PCR products obtained at 35 cycles are shown. This experiment was repeated three times with similar results. Re-Chip assay: precleared chromatin samples, immunoprecipitated with anti-ARA70 and eluted as described under "Experimental Procedures," were re-immunoprecipitated with anti-ERα antibody or normal mouse IgG in negative control (*lane 3*). The new immunocomplexes were processed as in the ChIP assay, and the *pS2* promoter region was amplified by PCR using specific primers.

at the higher ectopic expression of AR potentiates the down-regulatory effect on ERα transcriptional activity (Fig. 8*B*). These data suggest that ARA70, present in limiting cellular amounts, may switch into the opposite effects of enhancing either the ERα or AR transcriptional activities according to the ratio between the two receptors in the cells.

ARA70 Interaction with Either AR or ERα Is Related to the Intracellular Content of Both Steroid Receptors—To investigate if ARA70 is able to interact with AR and/or ERα in relationship to their intracellular levels, coimmunoprecipitation assays were performed in HeLa cells cotransfected with both ERα and AR in a ratio of ERα/AR = of 5:1 or at a ratio of ERα/AR = of 1:5 in the presence of an excess of ARA70. Coimmunoprecipitation assay was carried out by using an anti-ARA70 antibody for immunoprecipitation followed by Western blot analysis of ERα. The nitrocellulose filter was then stripped and blotted with anti-AR antibody.

As indicated in Fig. 9*A* in the presence of an excess of ERα (ratio ERα/AR = 5:1), ARA70 predominantly coimmunoprecipitates with ERα. By contrast, when AR content is higher than ERα (ratio ERα/AR = 1:5), ARA70 coimmunoprecipitates exclusively with AR. Similar results were obtained in MCF-7wt expressing high levels of endogenous ERα and in MCF-7 overexpressing AR (MCF-7/AR) (Fig. 9*B*).

In MCF-7wt, expressing low levels of AR, ARA70 coimmunoprecipitates exclusively with ERα. In these cells, AR overexpression induces a prevalent interaction between ARA70 and AR. Moreover, in these experimental conditions and under estradiol treatment, ERα-ARA70 interaction is no longer detectable, because in these circumstances endogenous ERα expression is dramatically down-regulated. In addition in MCF-7 cells overexpressing AR, under E_2 treatment, ChIP assay demonstrates a significant decrease in the ARA70 occupancy of the estrogen-responsive region of the *pS2* promoter (Fig. 9*C*).

ARA70 Participates in the Down-regulation of ERα Expression Induced by Overexpressed AR—To examine whether ARA70 is involved in the down-regulation of ERα content, we analyzed the expression of ERα in MCF-7/AR cells expressing a dominant negative ARA70.

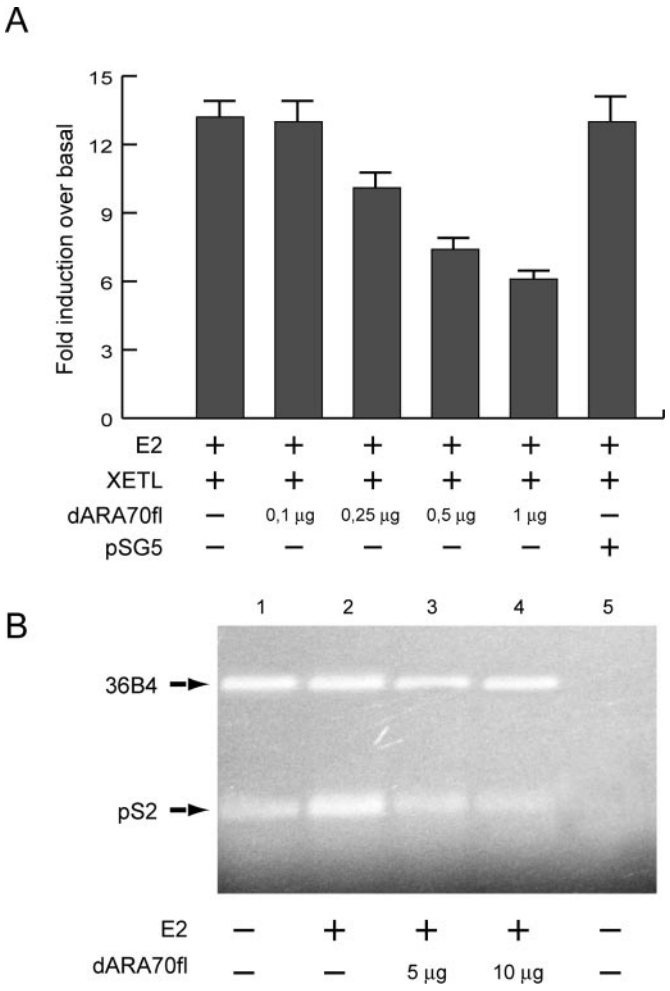

FIG. 6. **The dominant negative (dARA70fl) inhibits ERα transactivation in MCF-7 cells as well as *pS2* mRNA expression.** *A*, MCF-7 cells were transiently cotransfected with XETL reporter plasmid (0.5 µg/well) either in the absence or presence of pSG5 control vector or increasing amounts of dARA70fl expression plasmid, as indicated. After transfection cells were left untreated or treated with 10^{-7} M E_2 and harvested after 18 h, and luciferase activities were determined. In absence of E_2, no significant level of XETL luciferase activation was observed. Results represent the means ± S.E. of three separate experiments each in duplicate. *B*, MCF-7wt cells transiently transfected with either pSG5 control vector or increasing amounts of dARA70fl expression plasmid, as indicated (µg/10-cm dish), were left untreated or treated with 10^{-7} M E_2 for 24 h. Total RNA was isolated and reverse-transcribed. The first strand cDNA was subsequently subjected to semi-quantitative PCR using *pS2*-specific primers. *Lane 5*, MCF-7 RNA sample without the addition of reverse transcriptase (negative control). 36B4 mRNA levels were determined in the same amplification tube as control.

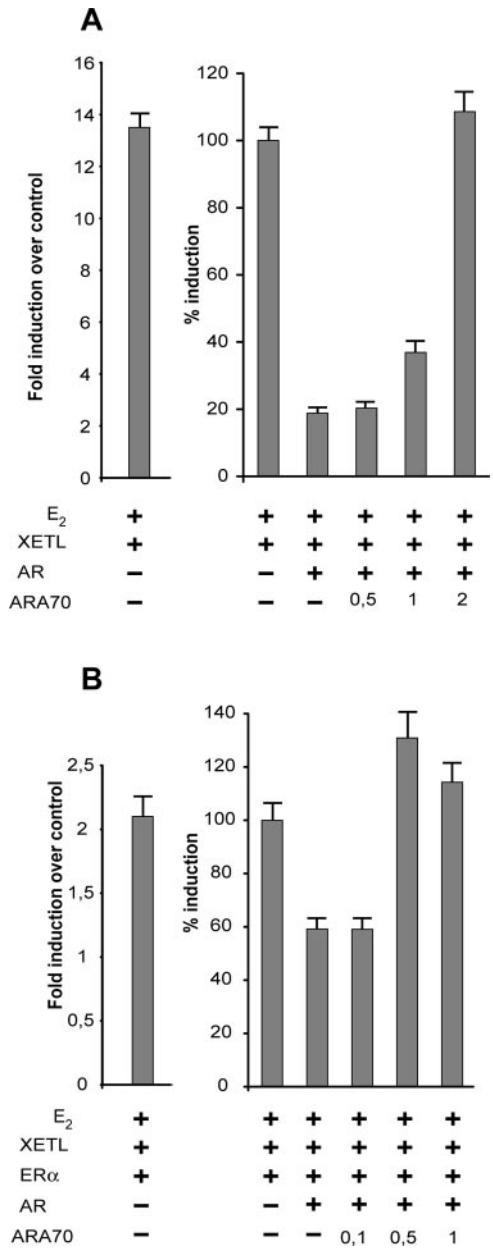

FIG. 7. **Exogenous expression of ARA70 abrogates AR-mediated repression of ERα transcriptional activity in both MCF-7 and HeLa cells.** *A*, MCF-7 cells were transiently cotransfected with XETL reporter plasmid (0.5 µg/well) either in the absence or presence of AR full-length expression plasmid (0.5 µg/well) and increasing amounts (given in µg/well) of full-length ARA70 expression plasmid, as indicated. *B*, HeLa cells, expressing ectopic ERα (0.5 µg/well), were transiently cotransfected with XETL reporter plasmid (0.5 µg/well) either in the absence or presence of AR full-length expression plasmid (0.1 µg/well) and increasing amounts (given in µg/well) of full-length ARA70 expression plasmid, as indicated. After transfection, cells were left untreated or treated with 10^{-7} M E_2 and harvested after 18 h, and luciferase activities were determined. In the absence of E_2, no significant level of XETL luciferase activation was observed. Activation of reporter gene expression XETL in the presence of E_2 is arbitrarily set at 100%. Results represent the means ± S.E. of three separate experiments each in duplicate.

To that end, MCF-7 cells were transiently transfected with plasmid encoding ARA in presence or absence of full-length dominant negative ARA70 and either treated or left untreated with 10^{-7} M of E_2. As shown in Fig. 10, the down-regulation of ERα induced by the overexpressed AR is partially reversed in presence of the dominant negative ARA70.

In addition to investigating whether the overexpression of ARA70 in MCF-7/AR cells is able to restore ERα content, we transiently cotransfected these cells with the ARA70 expression plasmid. In these experimental conditions no substantial change in ERα expression was observed with respect to MCF-7/AR cells (data not shown). These data indicate that in the presence of AR overexpression, ARA70 may be involved in the down-regulation of ERα content.

DISCUSSION

It is well known that the ERα expression in breast cancer is generally considered to represent a "good" prognostic marker for the disease outcome and anti-estrogen treatment responsiveness in breast cancer (22).

The activity of ERα is modulated by interaction with coregulators that are often shared with other members of the nuclear

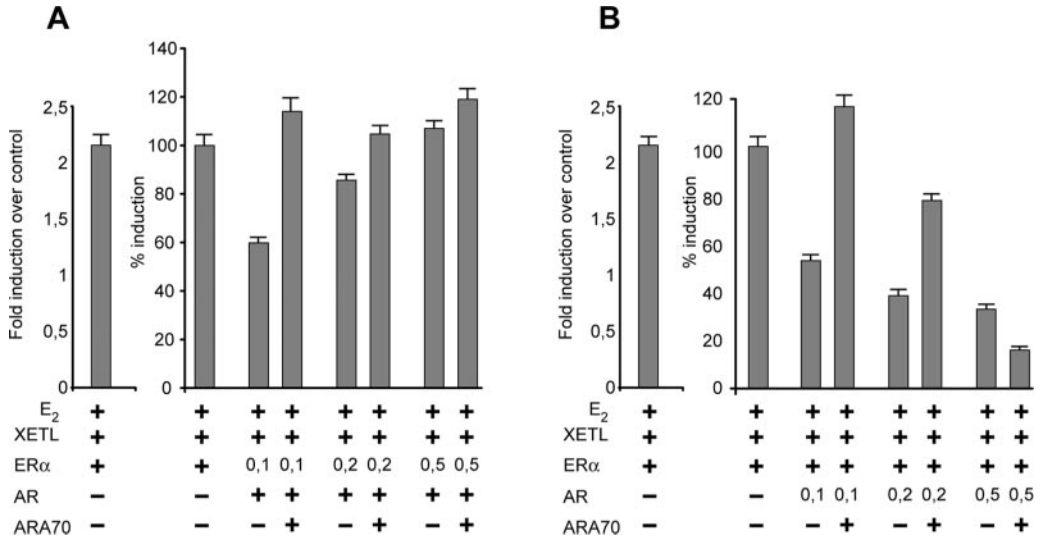

FIG. 8. **ARA70 modulatory effect on ERα transcriptional activity is related to AR and ERα content.** *A*, HeLa cells were transiently cotransfected with XETL reporter plasmid (0.5 μg/well) either in the absence or presence of AR full-length expression plasmid (0.1 μg/well) and ARA70 expression plasmid (1 μg/well) and increasing amounts of ERα expression vector (given in μg/well) as indicated. *B*, HeLa cells, expressing ectopic ERα (0.1 μg/well), were transiently cotransfected with XETL reporter plasmid (0.5 μg/well) either in the absence or presence of ARA70 expression plasmid (1 μg/well) and increasing amount of AR expression vector (given in μg/well) as indicated. After transfection, cells were left untreated or treated with 10^{-7} M E_2 and harvested after 18 h, and luciferase activities were determined. In the absence of E_2, no significant level of XETL luciferase activation was observed. Activation of reporter gene expression XETL in the presence of E_2 is arbitrarily set at 100%. Results represent the means ± S.E. of three separate experiments each in duplicate.

receptor superfamily and are able to stimulate or inhibit activation of target genes (23). Moreover, both coregulators and ERα expression may also be affected by multiple cell signaling pathways concomitantly present during tumor progression (24, 25). Therefore, understanding the role of all these cofactors that link E_2/ERα signaling to cell transcription machinery is critical to further develop new therapeutic approaches and to identify new targets for prevention and treatment of human hormone-dependent breast cancer.

In this scenario, it becomes intriguing to investigate the mechanisms through which the AR antagonizes ERα signaling in breast cancer. Previous studies indicate that the most powerful androgen DHT inhibits basal and estrogen-induced proliferation in ZR-75–1 and MCF-7 breast cancer cells through activation of the AR (9, 26). Moreover, many epidemiological studies have demonstrated that over 70% of human breast cancer biopsies from untreated women are positive for AR; the percentages are usually higher than or equally high as the percentages of ER and PR positivity (3, 27).

These observations led us to investigate the mechanism involved in the inhibitory role of androgen receptor on estrogen-regulated growth in a human breast cancer cell line MCF-7. Our data show that prolonged MCF-7 cells exposure to DHT induces a marked increase of AR intracellular levels concomitantly to a decrease in ERα content. On the other hand, from our findings it emerges how the overexpression of AR *per se* is able to inhibit ERα transcriptional activity in MCF-7 cells.

This effect is mediated by AR since the addition of OHFl reversed it (data not shown). This effect appears to be mediated at the genomic level as in MCF-7 cells, transiently transfected with an androgen receptor mutated in the DBD, no substantial changes were observed in estradiol-induced signal. By contrast, the ectopic expression of a constitutively active AR (AR-(1–707)) lacking the LBD results in the inhibition of an estradiol-induced signal which, as expected, still persists even in the presence of the androgen antagonist OHFl.

The above data suggest that the inhibitory effect of DHT on estrogen-dependent MCF-7 cells proliferation may occur through mechanisms that involve two potentially distinct effects: 1) down-regulation of ERα content and 2) reduced ERα transcriptional activity. The capacity for the activation of transcription by one nuclear receptor to compromise the transcriptional response dependent on a second receptor implies that shared components of transcriptional machinery are involved (10), suggesting that AR and ERα might use a common pool of factors present in limiting cellular concentrations.

The ability of AR in inhibiting the growth of breast cancer cells and ERα transcriptional activity led us to explore the role of a cofactor, ARA70, first described as an AR-specific coactivator (12). Expression of this factor has been reported to be decreased in prostate cancer and breast cancer with respect to the non-tumor tissues (21). This interest has been further heightened by a recognition that other events involved in breast cancer genesis or progression have been shown to alter AR expression or function.

In BRCA1-mutated tumors, loss of AR expression, and thus loss of AR signaling, supports neoplastic transformation of mammary epithelial cells harboring a BRCA1 mutation (28). Moreover, in HER2-positive breast cancers, generally associated with a worse outcome, a trend toward a loss of AR and/or ARA70 has been demonstrated (29). For this reason, we hypothesized that in breast, loss of AR and/or ARA70 protein expression might confer a growth advantage to cancer cells. In other words, ARA70 might participate in the interplay between AR and ERα in a manner crucial to regulating the proliferation of breast epithelial cancer cells and tumor progression.

In this study we demonstrated that ARA70 is a coactivator for ERα. We found that the transcriptional activity of the endogenous ERα in MCF-7 breast cancer cells is increased when ARA70 is overexpressed. The effect of ARA70 on ERα function is the result of physical interaction between endogenously expressed ERα and ARA70 as suggested by the data obtained in the coimmunoprecipitation assay. Moreover, the ChIP assay demonstrated that estrogen treatment induces an increase in the occupancy of the promoter of a classically estrogen-regulated gene, *pS2*, by endogenous ARA70 in MCF-7 cells. On the other hand, the functional role of ARA70 in enhancing ERα transactivation is further demonstrated by the evidence that in the presence of dominant negative ARA70, ERα transcriptional activity as well as *pS2* mRNA were dras-

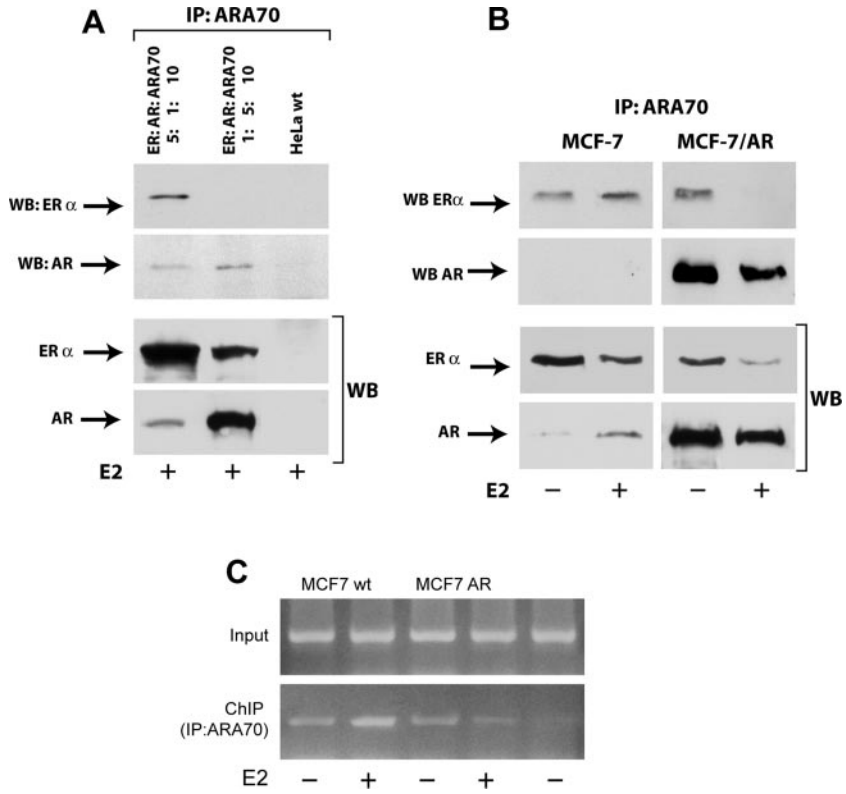

FIG. 9. **ARA70 interaction with AR and/or ERα is related to the intracellular content of the two steroid receptors.** *A*, *upper panel*, HeLa cells were transfected with the following: *lane 1*, ERα expression plasmid (5 μg/dish), AR expression plasmid (1 μg/dish), and ARA70 expression plasmid (10 μg/dish) as indicated; *lane 2*, ERα expression plasmid (1 μg/dish), AR expression plasmid (5 μg/dish), and ARA70 expression plasmid (10 μg/dish) as indicated. HeLa cells wt were used as negative control (*lane 3*). After a 48-h culture in the presence of 10^{-7} M E_2, cell extracts were prepared and subjected to immunoprecipitation with goat anti-ARA70 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblotting with mouse anti-ERα antibody. Nitrocellulose filter was then stripped and blotted with mouse anti-AR antibody. *Lower panel*, an aliquot of cell extracts was subjected to immunoblotting with specific antibodies, without prior immunoprecipitation, as indicated. *WB*, Western blot. *B*, *upper panel*, cell extracts from MCF-7wt and MCF-7 overexpressing AR (5 μg/dish) were prepared and subjected to immunoprecipitation with goat anti-ARA70 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblotting with mouse anti-ERα antibody. Nitrocellulose filter was then stripped and blotted with mouse anti-AR antibody. *Lower panel*, an aliquot of cell extracts was subjected to immunoblotting with specific antibodies, without prior immunoprecipitation, as indicated. *C*, soluble, precleared chromatin was obtained from MCF-7wt and MCF-7 (MCF-7/AR) transfected with 22 μg/dish of AR expression plasmid, treated with 10^{-7} M E_2 for 1 h (+) or left untreated (−), and immunoprecipitated with anti-ARA70 antibody or with normal goat IgG as negative control (*5th lane*). *pS2* promoter sequences containing ERE were detected in the recovered DNA by PCR amplification with specific primers. To determine input DNA, the *pS2* promoter fragment was amplified from 5 μl of purified soluble chromatin before immunoprecipitation. PCR products obtained at 35 cycles are shown. This experiment was repeated three times with similar results.

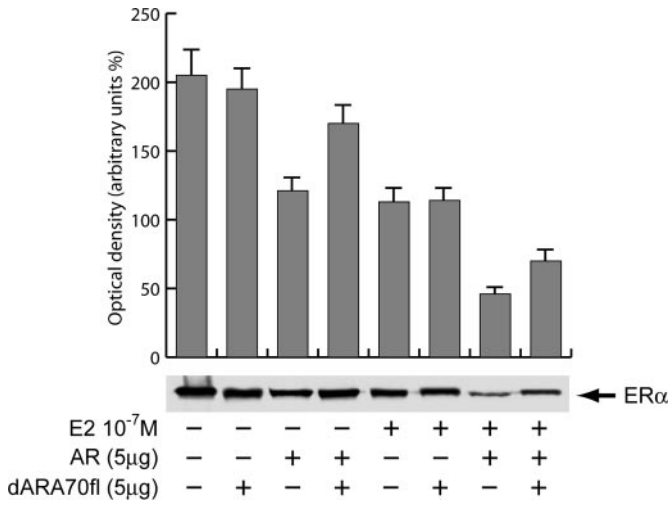

FIG. 10. **ARA70 is involved in the down-regulation of ERα content.** MCF-7 cells were transiently cotransfected as indicated and grown in the absence or presence of E_2 for 48 h. Whole cells extracts were prepared and subjected to immunoblotting with mouse monoclonal anti-ERα antibody. β-Actin was used as loading control. The *upper panel* shows the quantitative representation of data (mean ± S.E.) of three independent experiments after densitometry.

tically down-regulated. However, our results do not distinguish whether ERα-ARA70 interaction is direct or whether it is mediated by another yet unknown factor assembling a multiprotein complex with ARA70 and ERα.

Thus, ARA70 is a shared AR/ERα coactivator and therefore the transcriptional interference between AR and ERα might be mediated through competition for limiting amounts of ARA70 in the cell. Our data show that exogenously expressed ARA70 reverses the AR-induced inhibition of ERα transcriptional activity in both MCF-7 or HeLa cells. In addition, in HeLa cells, in the presence of a progressive increase of exogenously expressed AR, ARA70 is no longer able to restore an estradiol-induced signal, and at the maximal induced AR expression, it appears to synergize to AR in a down-regulating ERα transcriptional activity.

The ability of ARA70 to modulate AR/ERα interplay seems to be dependent on the steroid receptor content expressed in a specific cell type. In transiently transfected HeLa cells, coimmunoprecipitation assay indicates that in presence of an excess of ERα (ratio ERα/AR = 5:1) ARA70 prevalently coimmunoprecipitates with ERα. In contrast, when AR content is higher than ERα (ratio ERα/AR = 1:5) ARA70 coimmunoprecipitates exclusively with AR.

Most interestingly, similar results were obtained in intact

MCF-7wt and MCF-7 overexpressing AR (MCF-7/AR). It is worth noting that in this cell line, upon E_2 treatment and in the presence of the ectopically overexpressed AR, no apparent co-immunoprecipitation between ARA70 and ERα can be observed, due to the induced strong decrease of endogenous ERα. Moreover, the ChIP assay indicates that in MCF-7 overexpressing AR, under E_2 treatment, a significant decrease in the ARA70 occupancy of the estrogen-responsive region of the *pS2* promoter can be observed. Additional analysis of the expression of ERα in MCF-7/AR cells expressing a dominant negative ARA70 indicates that ARA70 coactivator is involved in the reduced ERα content that is observed in these cells.

These data, together with the functional data demonstrating that in these circumstances ERα activation is strongly inhibited, led us to conclude that the overexpression of AR in breast cancer cells, through ARA70, may reduce any stimulatory effect induced by E_2.

In conclusion, our results indicate that ARA70 is a cofactor able to alter the E_2/ERα signal in two ways, according to its recruitment by either ERα or AR. The alternative possibility of binding either nuclear receptor is tightly dependent on their intracellular concentration. It remains to be clarified whether the role of ARA70 is distinct from that of other important cofactors particularly expressed in breast cancer cells, like AIB1 (30, 31), as well as whether the ARA70 modulatory effect on ERα signal may be potentially mediated by such a cofactor.

Nonetheless, our study demonstrates that alteration in the expression levels of ARA70 is able to modulate the effects of AR on ERα signaling pathways. It is not yet clear whether this modulation is the consequence of direct interactions (*e.g.* alterations in the interaction of ARA70 with the AR and ERα) or whether the changes in ARA70 expression are more indirectly affecting the protein-protein interactions that are required for the normal function of the ERα in MCF-7. Discerning the importance of such direct and indirect effects will form the basis of future investigations.

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Endogenous Coactivator ARA70 Interacts with Estrogen Receptor α (ERα) and Modulates the Functional ER α/Androgen Receptor Interplay in MCF-7 Cells

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