ABSTRACT While all three coactivators ARA70, steroid receptor coactivator 1, and RAC3/ACTR can enhance androgen receptor (AR) transcriptional activity at 1 nM dihydrotestosterone, we here demonstrate that only ARA70 can induce AR transcriptional activity >30-fold in the presence of 10 nM 17β-estradiol (E2), but not diethylstilbestrol. The significance of this newly described E2-induced AR transcriptional activity in DU145 human prostate cancer cells was further strengthened by finding patients with Reifenstein partial-androgen-insensitive syndrome that fail in the E2-AR-ARA70 pathway. Together, our data suggest, for the first time, testosterone/dihydrotestosterone may not be the only ligands for the AR. E2 represents another important natural ligand for AR that may play an essential role for the AR function and the development of the male reproductive system.

The androgen receptor (AR) belongs to the steroid receptor superfamily that function primarily as transcription factors to regulate the expression of target genes by binding to specific hormone-responsive elements. These steroid receptors may exist within the target cells in a nonactivated state (1). After binding of the ligand, the activated receptors may interact with the hormone-responsive element, and the complex communicates with the transcriptional apparatus of the cell to induce or repress the expression of their target genes. It appears likely that coactivators or corepressors may be involved in this transactivation process and may function as a bridge between the receptor and the basal transcriptional factor complex (2, 3). Indeed, several coactivators or corepressors have been identified that may act as mediators to regulate the transactivation of the steroid receptors (4–12). As reported previously, we have successfully used the C-terminal domain of AR as bait to isolate the first AR coactivator, ARA70 (12). In the current report, we present evidence to demonstrate that E2, but not diethylstilbestrol (DES) or other estrogens, can activate androgen target genes via its interaction with the AR-ARA70 complex. The results also argue strongly that in addition to the AR itself, the presence of ARA70 is an important factor for maximal induction of E2-mediated AR transcriptional activity. Together, our data suggest the specificity of estrogen vs. androgen may be conferred via the proper interaction of AR and ARA70.

MATERIALS AND METHODS

Chemicals and Materials. The special purified 17β-E2 without androgen contamination was from Steroids, Inc. (Wilton, NH; with purity certification). Two other batches of highly purified 17β-E2 (purity > 99.8%) were from Sigma, and Steroids, Inc. All three 17β-E2 generated similar and reproducible results in our transfection assay. 17α-E2, DES, tamoxifen (Tam), and dexamethasone (Dex) were also from Sigma. ICI 182,780 (ICI) was kindly provided by A. Wakeling (Zeneca, Wilmington, DE).

Yeast and Mammalian Two-Hybrid Assay. In the interaction growth assay, the AR bait and ARA70 cotransformed yeast Y190 cells were selected on plates with 25 mM 3-aminotriazole and a serial concentration of different hormones, but without histidine, leucine, or tryptophan (12). The toxicity of different hormones, dihydrotestosterone (DHT), Dex, 17α-E2, 17β-E2, and DES (from 10⁻⁹ to 10⁻⁵ M), to the yeast growth has been tested with general nutrition supply, and the growth variances are within 20%. The mammalian two-hybrid system used in our test system mainly follows the protocol of CLONTECH, with some modifications. To obtain better expression, the GAL4 DBD (amino acids 1–147) was fused to pSG5 that was driven by simian virus 40 promoter, and named GAL0. The hinge and ligand binding domain of wild-type AR and mutant ARe70k were then inserted into GAL0, respectively. Similarly, the VP16 activation domain was fused to pCMX, which was driven by cytomegalovirus promoter, and named pCMX-VP16 (provided by R. M. Evans, The Salk Institute for Biological Studies, La Jolla, CA). This pCMX-VP16 was then used to construct fusion of ARA70.

Abbreviations: AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; PR, progesterone receptor; DES, dihydrotestosterone; DHT, dihydrotestosterone; CAT, chloramphenical acetyltransferase; E2, 17β-estradiol; MMTV, mouse mammary tumor virus; Dex, dexamethasone; Tam, tamoxifen; ICI, ICI 182,780.
Transient Transfection and Chloramphenical Acetyltransferase (CAT) Assay. In each transfection, 3.5 \( \mu \)g of mouse mammary tumor virus (MMTV)-CAT was used as reporter. One and one-half micrograms of hAR with or without 4.5 \( \mu \)g ARA70 or other cofactors were transfected into DU145 cells. Relative CAT activity was calculated by PhosphorImager quantification.

\( \beta \)-Galactosidase activity was used to normalize transfection efficiency and to indicate cell viability under different concentrations of ligand treatment.

**Ligand Binding Assay.** Binding of \( [3H]E2 \) (DuPont yNEN) was measured by the hydroxyapatite filter method (19). The result was expressed as a percentage of the label bound in the control tube (4,000 cpm) without additional unlabeled steroid. The experiment was repeated three times to assure steroid specificity.

**RESULTS**

**Effects of Estrogens on the Interaction of Wild-Type AR and ARA70 in Yeast and Human Prostate Cells.** Using a yeast two-hybrid system under a serially diluted concentration of different hormones, we found that 50 nM E2 could induce an interaction between ARA70 and GAL4DBD-AR, but DES (a more potent, nonsteroidal estrogen) was unable to promote this interaction even at pharmacological concentrations (10\(^{-6}\)–10\(^{-5}\) M, Fig. 1A shows the results of 10\(^{-5}\) M). Other nonandrogenic steroids such as Dex and 17\(\alpha\)-E2 also did not induce any significant interaction (Fig. 1A). The specific dose-dependent E2 induced AR-ARA70 interaction (starting at physiological concentration 1 nM E2) was also demonstrated in the mammalian two-hybrid assay (Fig. 1B, lanes 4–7). Together, these data indicate that the E2-mediated physical association between AR and ARA70 exists both in yeast and mammalian cells.

**Effects of E2 on the AR Transcriptional Activity in the Presence of ARA70.** Using DU145 cells, our MMTV-CAT assay data further showed that 1 nM E2 can start to induce transcriptional activity of AR (reaching >30-fold increase at 10 nM E2) only in the presence of ARA70 (Fig. 1C). DES and other estrogens/antiestrogens, such as estrone, 17\(\alpha\)-E2, estriol, Tam, and ICI, showed very little induced activity, even at pharmacо-
logical concentration of $10^{-6}$M (Fig. 1C). A similar induction pattern mediated by E2 also occurred when we replaced MMTV-CAT with another androgen target gene, prostate-specific antigen-CAT (data not shown). As the contamination of androgen in the E2 used in our assay is not an issue (see Materials and Methods), these data strongly suggest that ARA70 may represent an important cofactor for the E2-mediated AR transcriptional activity.

The E2-Mediated Induction of AR Transcriptional Activity in the Presence of ARA70 Is Not Via Estrogen Receptor (ER), Progesterone Receptor (PR), or Glucocorticoid Receptor (GR). It is well documented that AR, GR, and PR could recognize the same consensus sequence. To rule out the possibility that E2-induced MMTV-CAT activity in the presence of ARA70 was mediated by other steroid receptors, such as PR, GR, or ER, we replaced AR with these receptors in our MMTV-CAT assay. As shown in Fig. 2A, our data demonstrate that only AR, but not ER, PR, or GR, can significantly induce MMTV-CAT activity in the presence of 1–10 nM E2 and ARA70 (Fig. 2, lanes 1–14). As there is no estrogen response element in MMTV promoter-CAT, E2 cannot activate ER activity. The inclusion of ER in this experiment is to prove the E2-AR-ARA70 pathway is not through E2-ER. Again, our data also indicate that ARA70 only slightly enhances the transactivation of PR and GR in this assay (Fig. 2, lanes 17–20). Thus, ARA70 is a relatively specific coactivator for AR, which is consistent with our previous report (12).

As AR or ER could also be activated by phosphorylation (20, 21), it is possible that E2 may bind and activate ER, which somehow triggers a kinase system to phosphorylate and to activate the AR in our system. However, two factors argue strongly against this hypothesis. (i) The E2 effect on the association of AR and ARA70 has been verified in the budding yeast Y190, Saccharomyces cerevisiae, which contains no endogenous ER (22). (ii) Other estrogenic compounds, including DES, a more potent estrogen than E2, cannot induce the association of AR and ARA70 in yeast nor the transcriptional activity of AR and ARA70 in DU145 cells (Fig. 1). Based on these two findings, we therefore believe that AR and ARA70, but not the ER, are the essential factors for E2-induced AR transcriptional activity.

While Fig. 1C suggests that DES, Tam, and ICI by themselves cannot induce any significant AR transcriptional activity at concentrations of $10^{-5}$–$10^{-6}$ M, Fig. 2B shows that at 2 × $10^{-5}$–$10^{-3}$ M (200- and 1,000-fold concentrations of $10^{-6}$M E2), DES, Tam, and ICI can repress E2-mediated induction of AR transcriptional activity. These data suggest that DES, Tam, and ICI may share some, but not all, E2 binding sites in the AR-ARA70 complex. A crystallography study of AR-ARA70 in the presence of E2 or DES should be able to answer this question.

**E2 Binding of AR and ARA70.** A cell-free in vitro transcription/translation system was used to test whether ARA70 can either bind to E2 or increase the AR binding to E2. As shown in Fig. 3A, [3H]E2 has 10% and 1% (using E2-ER as 100%) of the relative binding affinity between E2 and AR, respectively (lanes 15–20). DES, ICI, and Tam can inhibit the E2-mediated induction of AR transcriptional activity in the presence of ARA70. The induction of AR-ARA70 transcriptional activity by 10 nM E2 was counted as 100% (lane 2). The levels of increased inhibition relative to the concentration of E2 were shown as the following: ICI (lanes 3 and 4: 200-fold and 1,000-fold); Tam (lanes 5 and 6: 200-fold and 1,000-fold); DES (lanes 7 and 8: 200-fold and 1,000-fold).

**Materials and Methods.**

**E2-Ligand binding of AR and ARA70.** (A) In vitro synthesized AR, ARA70, and ER were quantitated by [35S]methionine labeling. Equal molar concentrations of ER and AR were used for the [3H]E2 ligand binding assay. Three-fold molar ARA70 was incubated with AR on ice for 1 hr before adding 50 nM [3H]E2. Two hundred-fold unlabeled E2 was used as a competitor to determine the specific binding. (B) E2-specific binding of full-length AR. AR was synthesized and translated in a rabbit reticulocyte lysate system. Aliquots of the lysate were then incubated with [3H]E2 (87 Ci/mmol; 1 Ci = 37 GBq) in the presence or absence of 20-fold, 200-fold, and 500-fold unlabeled steroids. The final incubation volume was 100 μL. The values of duplicate assay tubes were within 10% of the average shown in the figure.
ARA70 can induce AR activity from the concentration of 1 nM coactivators enhance DHT-AR transcriptional activity, only functionally enhance the transactivation of AR. While all three tors can enhance AR transcriptional activity at 1 nM DHT.

Although it has been speculated that SRC-1 and RAC3 are the coactivators for many steroid hormone receptors, this is also indicated that only DHT, but not E2, can promote the lost E2-induced AR activity (23). Mammalian two-hybrid system ARe708k maintained DHT-induced AR activity but somehow mutation at amino acid 708 (glutamic acid to lysine, named ARe708k) was identified and preliminary data suggested that ARA70 is the effective coactivator for E2-mediated AR transcriptional activity.

To investigate whether E2-AR-ARA70 transcriptional Activity. To extend our Mutant ARs in the Presence or Absence of ARA70. To our ligand binding assay suggests that, while the E2-AR binding is specific, ARA70 by itself does not bind E2, or increase the affinity of E2 binding to AR. However, it is possible that the classic ligand binding assay could not detect the subtle increase of E2-AR binding that is required for E2-induced AR activity.

**ARA70 Is the Effective Coactivator for E2-Mediated AR Transcriptional Activity.** To investigate whether E2-AR-ARA70 forms a special complex with a distinct conformation to mediate E2-induced AR activity, we compared three coactivators, SRC-1, RAC3/ACTR for their DHT- and E2-induced AR activity. As shown in Fig. 4A, all three coactivators can enhance AR transcriptional activity at 1 nM DHT. Although it has been speculated that SRC-1 and RAC3/ACTR are the coactivators for many steroid hormone receptors, this is the first evidence showing that SRC-1 and RAC3/ACTR do functionally enhance the transactivation of AR. While all three coactivators enhance DHT-AR transcriptional activity, only ARA70 can induce AR activity from the concentration of 1 nM E2 and shows >30-fold induction at 10 nM E2 (Fig. 4B). These data suggest that DHT-AR complex is sufficiently different from E2-AR that only ARA70 can confer the significant androgenic activity on E2.

**The E2-Mediated Induction of AR Transcriptional Activity on Mutant ARs in the Presence or Absence of ARA70.** To extend our E2-induced AR activity in DU145 cells in vivo, we first used the yeast mutagenesis system to screen for AR mutants that will not respond to E2-induced AR activity. An initial candidate with a mutation at amino acid 708 (glutamic acid to lysine, named ARe708k) was identified and preliminary data suggested that AR708k maintained DHT-induced AR activity but somehow lost E2-induced AR activity (23). Mammalian two-hybrid system also indicated that only DHT, but not E2, can promote the interaction of AR708k and ARA70 (Fig. 1B). Based on recent publications about the crystal structure of liganded and unliganded steroid receptor ligand binding domains, the ligand binding cavity is speculated to be formed by parts of helix 3 (H3), H4, H6, H11, H12, and the S1/S2 hairpin (24, 25). Our data further suggest that H3 is essential for the formation of ligand binding cavity. The change of the charge from glutamic acid to lysine on residue 708 is likely to influence the ligand binding. A further crystallography study of AR in the presence of E2 or DHT should be able to define the subtle structure differences of wtAR and mutant AR708k.

Suspecting that some patients with androgen response disorders may carry AR708k, we identified one Reifenstein syndrome patient with partial androgen insensitivity, who does indeed carry the AR708k mutation. As shown in Fig. 5, while this patient had abnormal male reproductive organs, he had normal testosterone and E2 concentrations. Primary cultures of genital skin fibroblasts from this patient also showed normal androgen binding capacity, Kd for DHT to AR, and 5α-reductase activity. Surprisingly, when we replaced the patient’s mutant AR708k with wild-type AR in the E2-AR-ARA70 MMTV-CAT assay, we found that AR708k has only slightly reduced DHT-mediated AR activity in the presence of ARA70 (Fig. 5C lane 3 vs. 13) or absence of ARA70 (Fig. 5C lane 2 vs. 12). The only significant difference in this patient’s androgen activity, as compared with normal, is that his mutated AR708k cannot enhance E2-mediated AR activity in the presence of ARA70 (Fig. 5C lane 6 vs. 16; Fig. 5D lanes 9 and 10 vs. 29 and 30). The consequence of losing this E2-AR-ARA70 pathway while maintaining the DHT-AR and DHT-AR-ARA70 pathways may likely be one of the explanations for the Reifenstein syndrome with partially abnormal male reproductive organs (Fig. 5B). The inclusion of a mutant AR (ARt877a, threonine to alanine) found in many prostate tumors is not only to demonstrate that E2-AR-ARA70 can also function at physiological concentrations (1 nM E2) in prostate tumor but also to test the widely accepted hypothesis that a single amino acid mutation of the AR can allow E2 to induce AR activity (16, 17). As shown in Fig. 5D, while ARt877a mutant may increase the potency of E2 (lanes 4 vs. 14 and 8 vs. 18), a mutated ARt877a alone exhibits a relatively small induction of its AR transcriptional activity by E2, and ARA70 is still required to maximize E2-induced AR activity. Furthermore, using transient transfection assay, we have investigated the E2-AR-ARA70 transcriptional activity in PC3 and CHO cells that express endogenous ARA70. Because these cells may have higher expression of endogenous ARA70, the cotransfection of ARA70 cannot significantly induce the activity, but we are able to demonstrate that antisense ARA70 can block 35–45% of E2-mediated AR activity (data not shown). Together, these data suggested that ARA70 plays important roles in the proper or maximal E2-mediated AR transcriptional activity.
FIG. 5. The E2-mediated induction of AR transcriptional activity on mutant ARs. The ARe708k was from the partial-androgen-insensitive syndrome patient. The ARt877a was from LNCaP and prostate cancer patient. Fixed amount of AR and ARA 70 were used in transfection. (A) A schematic representation of the helix 3 of AR, PR, and GR showing the location of mutant e708k. (B) The bioprofile of the partial-androgen-insensitive syndrome patient with mutant ARe708k and the physical defect in the exterior reproductive system of the patient. (C) Effects of DHT and E2 on the transcriptional activity of wild-type AR, and ARe708k in the presence or absence of ARA70 in DU145 cells. (D) Effects of E2 on the transcriptional activity of wild-type AR, mutant ARt877a, and ARe708k in the presence or absence of ARA70.
DISCUSSION
While mutated ARs become widely accepted as the explanation for why E2 may induce prostate-specific antigen in prostate cancer cells (16, 17), recent evidence demonstrating that E2 can activate AR-target genes, such as the MMTV-long terminal repeat or prostate-specific antigen, in the presence of wild-type AR and ARA70 at 1:3 ratio. These data, along with evidence from the comparison of three cofactors (Fig. 4), suggest that ARA70 is a key factor for the E2- and androgen-mediated induction of androgen target genes in prostate. Therefore, a drug designed to block the interaction of AR and ARA70 could have significant therapeutic and perhaps preventative value.

Even after several decades, surgical or medical castration combined with the administration of antiandrogens remains as the major treatment for disseminated prostate cancer. Estrogens are still at times used to repress androgens, and currently provide a very favorable cost profile compared with any other means of androgen ablation. Many clinical trials have suggested that E2 would be less effective than DES (26, 27) when used to treat prostate cancer. Our findings that E2, but not DES, can activate androgen-target genes in the prostate (Fig. 1) may, therefore, provide one explanation for this observation. Also, the fact that DES may block E2-mediated AR activity may attribute a new antiandrogenic function to DES (Fig. 2B). Our observation that E2 and DES can have different functions (only E2 can activate hormone function and selectivity. Our finding that E2 can control the response, and the interaction between ligand- and estrogenic compounds may play a role in the disruption of normal endocrine functions in humans and other animals (36), it will be interesting to know whether any environmental pollutants-estrogenic compounds also have some androgenic activity that may contribute to the disruption of the endocrine system.

In summary, the new E2-AR-ARA70 pathway found in human prostate cancer cells suggests that a special coactivator ARA70 may be able to modulate the sex hormone specificity. Moreover, E2 may represent an essential ligand of AR that plays an important role in the development and/or functioning of the male reproductive system. Further studies of this E2-AR-ARA70 pathway may therefore allow us to develop new hormonal therapies for the treatment of prostate cancer and other androgen-related disorders.

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