Mutations in the Helix 3 Region of the Androgen Receptor Abrogate ARA70 Promotion of 17β-Estradiol-Induced Androgen Receptor Transactivation

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Running Title: Specific residues are critical for E2-dependent androgen receptor transcriptional activity

Key Words: Androgen receptor, ARA70, E2, partial androgen insensitivity syndrome
Abbreviations: AR, androgen receptor; LBD, ligand-binding domain; DHT, 5α-dihydrotestosterone; CAT, chloramphenicol acetyltransferase; E2, 17β-estradiol; MMTV, mouse mammary tumor virus.
ABSTRACT

The influence of estrogen on the development of the male reproductive system may be interrupted in a subset of Partial Androgen Insensitivity Syndrome (PAIS) patients. PAIS describes a wide range of male undermasculinisation resulting from mutations in the androgen receptor (AR) or steroid metabolism enzymes that perturb androgen-AR regulation of male sex organ development. In this study, we are interested in determining if PAIS-derived AR mutants that respond normally to androgen have altered responses to estrogen in the presence of ARA70, a coregulator previously shown to enhance E2-induced AR transactivation. The wild-type AR (wtAR), and two PAIS AR mutants, AR(S703G) and AR(E709K), all bind to androgen and E2 and subsequently translocate to the nucleus. While ARA70 functionally interacts with the wtAR and the PAIS AR mutants in response to androgen, E2 only promotes the functional interaction between ARA70 and the wtAR, but not the PAIS AR mutants. ARA70 increases E2 competitive-binding to the wtAR in the presence of low level androgen and also retards E2 dissociation from the wtAR. ARA70 is present in both the cytoplasm and the nucleus of various mouse testicular cells during early embryogenesis day 16 and Postpartum day 0 during estradiol synthesis and of the Leydig cells at Postpartum day 7 weeks. ARA70 may be unable to modulate the PAIS AR mutants-E2 binding, diminishing the effect of E2 via AR during male reproductive system development in patients with such mutations. Therefore, the presence of ARA70 in the testosterone and estradiol-producing Leydig cells may enhance the overall activity of AR during critical stages of male sex organ development.
INTRODUCTION

Androgen action via the androgen receptor (AR) is critical in regulating male reproductive system development (1). Insufficient androgen action may cause androgen insensitivity syndrome or male undermasculinised genitalia (2). The androgen insensitivity phenotypes vary from external genitalia that are completely female to degrees of partial masculinisation. The possible causes may range from mutations in the AR or 5α-reductase genes to abnormal levels of estrogen vs. androgen. While partial androgen insensitivity (PAIS)-associated AR mutations usually disrupt the response to androgen, a subset of patients has AR mutations without any apparent defect in androgen signaling (3,4).

Estrogen, primarily 17β-estradiol (E2), has also been proposed to be involved in both normal and abnormal processes of male reproductive development and associated diseases (5,6). Male estrogen is synthesized by aromatization of androgen, (e.g. testosterone), in many tissues including brain, liver, adipose tissue, and prostate (5). The physiological level of circulating E2 in the adult male is approximately 0.1 nM (73-184 pmol/L or 12-34 pg/ml), and in the adult female during pregnancy, the level of E2 is approximately 1 nM (367-1285 pmol/L or 100-350 pg/ml) (7). However, local aromatase activity may cause particular tissue levels of E2 to be higher than the serum level. During pregnancy, maternal exposure to exogenous estrogen may influence the sexual maturation of male offspring, leading to cryptorchidism, epididymal cysts, and retained Mullerian ducts (8). Although estrogen action on the male reproductive system is not well understood, there is evidence of an estrogen imprinting effect that may induce prostatic hyperplasia in male offspring with excess prenatal exposure to estrogen (9).
Based on reports using CV-1 (10,11) or PC-3 (12) cells, it is possible that estrogen may influence male reproductive system development via the AR. Our previous report (13) demonstrated that E2-mediated wild type AR (wtAR) transactivation is enhanced by the addition of the AR coregulator ARA70, in DU145 cells. In HeLa cells, while E2 could promote the translocation of the wtAR from the cytoplasm to the nucleus, E2 requires selective coregulators, such as ARA70 or SRC-1, to induce significantly the wtAR transactivation (14). In PC-3 (15), CV-1 (16), and TSU-Pr1 (17) cells, ARA70 and β-catenin also dramatically enhance wtAR transactivation in response to E2, although there are conflicting results in CV-1 cells (16,18). The differing results in CV-1 cells may reflect differences in cell passage number, growth conditions, or expression vectors. Therefore, certain coregulators may enable estrogen to influence AR target gene expression via the AR, and thereby potentially contribute to male sexual maturation. Furthermore, there is evidence that the disruption of coactivators may also contribute to hormone resistance during target organ development (19-21). Thus, certain PAIS-associated AR mutations may disrupt this estrogen-dependent AR transactivation, modulated by coregulators, during male reproductive system development.

Here we have analyzed the functional interaction of PAIS-derived AR mutants with ARA70 in response to androgen or E2. Using co-immunoprecipitation and mammalian two-hybrid assays, we demonstrate that E2 promotes the interaction of ARA70 with the wtAR, but not with the PAIS mutants. By various ligand binding analyses, we also demonstrate that ARA70 increases the E2 competitive-binding to the wtAR at the low level of androgen and retards the dissociation of E2 from the wtAR-LBD, but not from the PAIS mutants. ARA70 is present in various fetal mouse testicular cells during early embryogenesis day 16 and Postpartum day 0 and is also significantly present in the Leydig cells at Postpartum day 7 weeks. ARA70 may therefore be unable
to stabilize the binding of E2 to the PAIS AR mutants, diminishing the effect of E2 via the AR during male reproductive system development in these patients.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Plasmids*—E2 from Sigma Chemical Co. (St. Louis, MO), [3H]-R1881 (3H-labeled 17β-hydroxy-17α-methylestra-4,9,11-trien-3-one), s.a., 85 Ci/mmol, [3H]-E2 (3H-labeled-(2,4,6,7)17β-estradiol), s.a., 118 Ci/mmol from DuPont/NEN (Boston, MA), and [14C]-chloramphenicol from Amersham Corp. (Arlington Heights, Il), were purchased. Gal4AR-LBD contains the human AR LBD residues 624-919 (encoded by exons D-H) fused to Gal4 DNA-binding domain residues 1-147. VP16-AR contains the near full-length human AR residues 38-919 fused to the carboxyl-terminal of the VP16 transactivation domain residues 411-456. pSG5-ARA70 contains ARA70 amino acids 1-401 for optimum co-activation.

*Site-Directed PCR Mutagenesis*—Mutations were designed based on the phenotypes of complete (N705S, L707R) and partial (S703G, E709K) androgen insensitivity (4). pSG5-AR-S703G is a gift from Dr. Helmut Klocker (3).

*Cell Culture, Transfections, and Reporter Gene Expression Assays*—COS-1 monkey kidney cells, H1299 human lung cancer cells, and DU145 human prostate cancer cells were maintained in phenol red free Dulbecco’s modified Eagle’s medium containing fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate, with 5% CO2 at 37°C. Transfections and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (13). pCMV-β-gal was used as an internal control.
The CAT activity was quantitated by PhosphoImager analysis (Molecular Dynamics). Data are presented as a mean ± S.D. of three independent experiments.

**Ligand Binding Assay**—The ligand-competition assay was determined by the modified hydroxylapatite filter assay using H1299 cells as described (22). Transfected-intact cells were incubated for 1 h at 37°C with 20 nM [³H]-R1881 in the presence or absence of a 100-fold excess of unlabeled R1881 or E2.

The whole cell binding assays was used to determine the effect of ARA70 on AR ligand binding as described (23,24,25). DU145 cells were transfected with AR (1.5 µg) or AR (1.5 µg) and ARA70 (4.5 µg). Equal amounts of pSG5 vector (4.5 µg) were used to substitute for pSG5-ARA70 in the AR alone transfection. The ARA70 effect on the binding of R1881 to the wtAR was determined by incubating transfected cells with 0.1 to 20 nM [³H]-R1881 in the presence or absence of a 100-fold excess of unlabeled R1881. The ability of unlabeled E2 to compete with [³H]-R1881 was determined by incubating transfected cells with 1 pM [³H]-R1881 in the presence or absence of various concentrations of unlabeled competitor E2. At the final stage, the cells were extensive washed with PBS and harvested. [³H]-R1881 bound of the crude protein lysate was quantitated using a scintillation counter. Specific binding was determined as the difference of [³H] counts between in the absence and in the presence of unlabeled ligand. Data are presented as a mean ± S.D. of three independent experiments.

**Immunostaining Assay**—COS-1 cells were transiently transfected using FuGENE6 transfection reagent (Boehringer-Mannheim) with pSG5 wtAR or various mutant ARs (2 µg), and treated with 10 nM DHT, or E2, or with vehicle. The AR was detected by an AR antibody (NH27) and visualized by goat anti-rabbit rhodamine-fluorescence (ICN). DAPI
staining was used to visualize the cell nuclei. The slides were photographed by confocal photomicroscopy, Magnification x400.

*Mammalian Two-hybrid Assay*-DU145 cells were co-transfected with Gal4-AR-LBD 624-919, VP16-ARA70 and the pG5-CAT reporter plasmids. pCMV-β-galactosidase was used as an internal control. Data are presented as a mean ± S.D. of three independent experiments.

*Co-immunoprecipitation*-COS-1 cells were transfected with vector alone or the wtAR and ARA70 at a 1:3 ratio. Cells transfected with wtAR and ARA70 were treated with 10 nM DHT, 10 nM E2, or vehicle. Protein lysate was then used for immunoprecipitation of wtAR with the AR antibody NH27. The wtAR and ARA70 were then resolved by Western blotting using AR (NH27) and ARA70 (CC70) antibodies and alkaline phosphatase-conjugated secondary antibodies (Bio Rad Laboratories).

*E2 Dissociation Assay*-DU145 cells were transfected with pSG5AR (1.5 µg) in the presence or absence of ARA70 (4.5 µg). After 24 hours, the transfected cells were treated with 0.1 nM [3H]-R1881 or 0.1 nM [3H]-E2 in the presence or absence of 100-fold excess of unlabeled R1881 or E2, and incubated for 2 h at 37°C in a 5% CO2 incubator. The cells were then washed extensively with PBS and incubated in the presence or absence of a 10,000-fold molar excess of unlabeled R1881 or E2 (23,24,25). The cells were harvested at the various time points. Crude cell lysate containing the receptor was determined for the radioactivity using a scintillation counter. Data are presented as a mean ± S.D. of three independent experiments.
**E2 Association Assay**—The transfected DU145 cells were incubated with medium containing 0.1 nM [³H]-E2 at 37 °C. The cells were harvested at various times within the first 10 minutes and at longer times (30, 60, 90 or 120 minutes) for the measurement of maximum binding. Next, the cells were washed extensively with PBS, and the reaction was blocked with a 100-fold excess of unlabeled ligand. The cells were harvested at the various time points. The cell lysate was determined for the radioactivity using a scintillation counter. Data are presented as a mean ± S.D. of three independent experiments.

**Immunohistochemical Assay**—Immunohistochemical localization of ARA70 and AR was conducted using the Vectastain Elite ABC Kit, and the Peroxidase Vector M.O.M.™ Immunodetection Kit (Vector Laboratories, Inc. Burlingame, CA, PK-2200). Paraffin-embbedded tissue from embryonic day 16 (E16), Postpartum day 0 and 7 weeks wild-type male mice was deparaffinized with xylene and rehydrated through a graded alcohol series. Briefly, 3% hydrogen peroxide was used to block endogenous peroxidase activity. Sections were incubated in the M.O.M.™ Mouse Ig Blocking Reagent to decrease non-specific binding and then incubated with mouse monoclonal AR (G122-77, Pharmingen International) (1:100) or ARA70 (CC70) (1:100) antibodies. Mouse IgG (Santa Cruz) was used as a control. Detection of primary antibody binding was conducted by incubation with M.O.M.™ Biotinylated Anti-Mouse IgG. Finally, Vectastain ABC reagent and peroxidase substrate solution were applied to the sections according to kit instructions. The sections were then counterstained with Gill's hematoxylin #2 to stain the nuclei.
Data Analysis-Pooled data are given as mean ± SD, and statistical significance was determined using Student's unpaired t test. Probabilities < 5% (p < 0.05) were regarded as significant.

RESULTS

ARA70 enhances the transactivation of wtAR, but not AR(S703G) or AR(E709K), in response to E2-We used PCR site-directed mutagenesis to create several AR mutants based on mutations within the helix 3 region of the AR-LBD found in patients with complete or partial androgen-insensitivity (4). Fig. 1A shows the positions of the mutations and a comparison of the helix 3 domains among the classic steroid receptors, including the AR, estrogen receptor (ER), glucocorticoid receptor (GR), and progesterone receptor (PR). All four amino acids, S703, N705, L707, and E709 are conserved between AR and PR. N705 and L707 are conserved among AR, PR, and GR. S703 and E709 are different among AR, GR and ER, suggesting that these residues may determine the receptor specificity for androgen, glucocorticoid, or estrogen.

We then tested the influence of the coregulator ARA70 on the transactivation of these mutants in response to either 10 nM DHT or 10 nM E2. AR negative DU145 cells were transfected with the MMTV(ARE)-CAT reporter (3.5 µg), and various ARs (1.5 µg) in the presence or absence of ARA70 (4.5 µg). The parent pSG5 vector (4.5 µg) was used to substitute for pSG5-ARA70 in the AR alone transfection. CAT activity was normalized according to β-galactosidase activity and fold CAT activity is expressed based on the induction fold relative to ethanol treatment (set as 1 fold). ARA70 did not promote the wtAR transactivation under vehicle treatment, which is consistent with our previous report (data not shown, 13). Fig. 1B shows that while ARA70 promotes DHT-dependent transactivation of the wtAR, AR(S703G), and AR(E709K) (lane 3), ARA70
only promotes E2-dependent transactivation of the wtAR (*lane* 5), but not of the AR(S703G), and AR(E709K) mutants. The AR(N705S) and AR(L707R) mutants showed no transactivation in the presence of ARA70, under either DHT or E2 treatment.

*Both androgen and E2 can bind to the wtAR, AR(S703G), or AR(E709K)*—Since E2 did not activate the transactivation of AR mutants in the presence of ARA70, we tested the binding ability of AR mutants for E2. Competitive binding assays were performed by using AR-negative H1299 cells transfected with the wtAR or the AR mutants, and incubating the cells with 20 nM [3H]-R1881 in the presence or absence of a 100-fold excess of unlabeled R1881, a synthetic androgen, at 37 °C for an hour. Fig. 2 shows that unlabeled R1881 competed with [3H]-R1881 for binding to the wtAR, AR(S703G) and AR(E709K) (*lane* 2). When we replaced unlabeled R1881 with unlabeled E2, we found that a 100-fold excess of unlabeled E2 can compete with [3H]-R1881 for binding to the wtAR, and the AR(S703G) and AR(E709K) mutants (*lane* 3). The AR(N705S) and AR(L707R) mutants showed no ability to bind [3H]-R1881 (*lane* 1). Similar results were also observed when we replaced H1299 cells with AR-negative COS-1 cells (data not shown).

These results suggest that both androgen and E2 can bind to the wtAR, AR(S703G), or AR(E709K). The abolished binding ability of AR(N705S) and AR(L707R) for [3H]-R1881 reflects the defective structure of the mutant receptor-LBD, and supports the importance of these conserved residues in the phenotype of complete androgen insensitivity syndrome. We also tested the expression level of the complete androgen insensitivity and PAIS AR mutants, and found a similar expression level of the AR mutants compared with wtAR (data not shown).
**E2 can promote nuclear translocation of wtAR, AR(S703G) and AR(E709K)** - We assayed the ability of AR(S703G) and AR(E709K) to translocate to the nucleus in response to E2 by immunocytofluorescence in COS-1 cells. As shown in Fig. 3A, in the absence of ligand, the wtAR and AR mutants are all localized in the cytoplasm. Addition of 10 nM DHT promoted the nuclear translocation of the wtAR, AR(S703G), and AR(E709K). Addition of 10 nM E2 also promoted the nuclear translocation of the wtAR, AR(S703G), and AR(E709K) (Fig. 3B). DAPI staining was used as a control for the location of the nuclei. Both E2 and DHT fail to bind to AR(N705S) and AR(L707R), and therefore cannot promote nuclear translocation of the receptors. Fig. 3C demonstrates the quantitative and statistical analysis of the E2-dependent nuclear localization of AR(S703G) and AR(E709K), compared to the wtAR using a total population of 50 transfected cells (n=50). The results indicate that E2 is able to promote the translocation of AR(S703G) and AR(E709K) into the nucleus.

Figs. 2 and 3 together suggest that E2, like DHT, can bind to AR(S703G) and AR(E709K) and promote their nuclear translocation. To confirm the results observed in the immunocytofluorescence experiments (Fig. 3B), we also applied Western blotting to detect the nuclear mutant AR level, in the presence or absence of 10 nM E2. We observed that stronger nuclear AR(S703G) and AR(E709K) signals were detected in the presence of 10 nM E2, compared to ethanol treatment (data not shown).

The results presented in Figs. 1 to 3 suggest that while E2 can bind and promote the nuclear translocation of the wtAR, E2 only slightly induced wtAR transactivation in the absence of ARA70. However, E2 can significantly induce wtAR transactivation in the presence of ARA70. Even though E2 can bind to and promote the nuclear translocation of the AR(S703G) and AR(E709K) mutants, E2 cannot activate these mutants, even in the presence of ARA70. Finally, the AR(S703G) or AR(E709K) mutations have no influence on DHT-mediated AR transactivation in the presence of
ARA70, suggesting that these residues may play a role in specifying E2- vs. DHT-dependent activation of AR, in the presence of ARA70.

*ARA70 directly interacts with the wtAR in the presence of E2 by co-immunoprecipitation assay.* We have previously demonstrated that E2 induces an interaction between VP16-ARA70 and Gal4DBD-AR using a yeast two-hybrid system (13). To confirm the direct E2-enhanced interaction between the wtAR and ARA70, we used a co-immunoprecipitation assay. Fig. 4A shows the direct E2-enhanced interaction of exogenous expressed wtAR and ARA70 in AR-negative COS-1 cells using an AR (NH27) antibody for co-immunoprecipitation, followed by Western blotting with AR and ARA70 antibodies.

*ARA70 does not interact with AR mutants in the presence of E2 by mammalian two-hybrid assay.* To understand why AR(S703G) and AR(E709K) are not activated by E2 in the presence of ARA70, we applied an *in vivo* mammalian two-hybrid interaction assay to determine the interaction between ARA70 and various ARs in the presence of 10 nM DHT or E2 in DU145 cells. Vehicle treatment did not promote the interaction between the wtAR or mutant and ARA70 (data not shown). As shown in Fig. 4B, pCMV-Gal4AR-LBD alone or together with the VP16 vector showed a low background level of receptor activity in the presence or absence of 10 nM DHT or 10 nM E2 (*lanes* 1 to 4). DHT can promote the interaction between ARA70 and the LBD of the wtAR, AR(S703G), or AR(E709K) (*lane* 5). E2 only promotes the interaction between ARA70 and the LBD of wtAR, but not AR(S703G) or AR(E709K) (*lane* 6). This loss of E2-promoted interaction between the mutant AR-LBDs and ARA70 may account for the lack of E2-mediated transactivation of AR(S703G) and AR(E709K) in the presence of
ARA70. As expected, the AR(L707R) mutant did not interact with ARA70 in the presence of DHT or E2 (lanes 5 and 6).

ARA70 influences the competitive-binding of E2 to the wtAR at the unsaturated androgen levels- We next tested the influence of ARA70 on R1881 binding to the wtAR. Fig. 5A shows that the equilibrium binding constant, Kd, of R1881-wtAR in the presence or absence of co-expressed ARA70. Kds for wtAR and wtAR with ARA70 were 2.13±1.22 and 1.82±1.21 nM, respectively. These results indicate that under equilibrium conditions, ARA70 has no effect on R1881 binding to the wtAR. Given the high affinity of the wtAR for androgen, its cognate ligand, it is not surprising that ARA70 has no significant effect on wtAR-androgen binding.

Under physiological conditions where both androgen and estrogen are present, it is clear that the specificity and affinity of the wtAR for androgen, is far higher than that for estrogen. During development, however, maternal estrogen may influence the balance between androgen and estrogen in the developing fetus. Therefore, we tested whether ARA70 influences E2 competitive-binding to the AR in the presence of unsaturated levels of androgen (Fig. 5B Upper). As shown in Fig. 5B Lower, [3H]-R1881 binding to the wtAR alone (set as a control) or together with ARA70 was similar in the absence of unlabeled E2 competitor (lanes 1, Left panel). In the presence of various concentrations of unlabeled E2 competitor, however, ARA70 co-expression significantly reduced [3H]-R1881 binding to the wtAR (lanes 2 to 6, Left panel). These data indicate that ARA70 may influence on E2 competitive-binding to the wtAR at unsaturated androgen levels. In contrast, ARA70 does not influence the competitive binding of E2 to the AR(E709K) (lanes 2 to 5, Right panel).
ARA70 retards the dissociation of E2 from the wtAR-The dissociation of [\(^3\)H]-E2 or [\(^3\)H]-R1881 from wtAR or AR(E709K) in the presence or absence of ARA70 was next assayed. We first determined [\(^3\)H]-R1881 bound for the AR alone or for the AR with ARA70. Fig. 6A shows a slight increase, however statistically insignificant, in [\(^3\)H]-R1881 binding to the wtAR cotransfected with ARA70 compared to the wtAR alone (lanes 3 vs. 1). After adding the 100X unlabeled E2 competitor, there was also a statistically insignificant variation, in [\(^3\)H]-R1881 binding to the wtAR cotransfected with ARA70 compared to the wtAR (lane 4 vs. lane 2).

We then tested the dissociation of [\(^3\)H]-R1881 from the wtAR alone vs. the wtAR cotransfected with ARA70 over 5 hours. Fig. 6B shows ARA70 did not substantially influence the dissociation of [\(^3\)H]-R1881 from the wtAR in the presence of excess unlabeled R1881 at 3, 4, and 5 hours, although [\(^3\)H]-R1881 dissociation was transiently reduced by wtAR cotransfected with ARA70 at early time. This transient reduction in [\(^3\)H]-R1881 dissociation in cells cotransfected with wtAR and ARA70 may be due to experimental variability that was evident in Fig. 6A (lanes 1 vs. 3 and 2 vs. 4). Moreover, in a dissociation assay, the later time points are more important, as a more prolonged effect on ligand dissociation will more greatly influence receptor transactivation. Therefore, ARA70 does not significantly affect [\(^3\)H]-R1881 dissociation from the wtAR, which is likely due to the higher affinity of androgen for the wtAR.

Similarly, we determined the influence of ARA70 on the dissociation of [\(^3\)H]-E2 from the wtAR. As shown in Fig. 6C, there was a slight variation, but not a statistically significant difference, of [\(^3\)H]-E2 binding to the wtAR cotransfected with ARA70 (lane 1 vs. 3). After adding 100X excess unlabeled E2, there was not a statistically significant difference between [\(^3\)H]-E2 binding to the wtAR and to the wtAR with ARA70 (lane 2 vs. 4). Over a 5-hour time period, however, ARA70 cotransfection did retard the dissociation of [\(^3\)H]-E2 from the wtAR in the presence of excess unlabeled E2 competitor.
(Fig. 6D). In contrast, ARA70 was unable to influence the dissociation of \(^{3}\text{H}\)-E2 from the AR(E709K). While Fig. 6E shows some variation, but not statistical significant, in \(^{3}\text{H}\)-E2 binding to the AR(E709K) in the absence (lane 1 vs. 3) or in the presence of unlabeled E2 (lane 2 vs. 4) with ARA70 cotransfection, Fig. 6F clearly indicates no difference in \(^{3}\text{H}\)-E2 dissociation over a five-hour period. These results suggest that the loss of the E2-dependent interaction between AR(E709K) and ARA70 prevents ARA70 from influencing E2 dissociation from this mutant.

**ARA70 does not influence the association of E2 with the wtAR** - We then tested whether ARA70 also influences the association of E2 with the wtAR or AR(E709K), using an *in vivo* ligand association assay in DU145 cells transfected with the wtAR or AR(E709K) with or without ARA70. Fig. 7A shows the similar acceleration of \(^{3}\text{H}\)-E2 binding to the wtAR compared to the wtAR cotransfected with ARA70. The similar association patterns are also observed between AR(E709K) alone and AR(E709K) cotransfected with ARA70 (Fig. 7B). Together Fig. 7A and B indicate that ARA70 does not influence the association of E2 with the wtAR or the AR(E709K) mutant.

**ARA70 is present in testicular Leydig cells during embryogenesis** - In a previous reports (26,27), the AR staining was found in a small number of interstitial cells in the primordial mouse testis from embryonic day 15 to 16 (E15 to E16). The AR was not obviously expressed in the Sertoli cells until postnatal day 5 (26). The AR was present in most cell types in the embryonic mouse testis at E16, and in germ cells and interstitial cells at Postpartum day 0 (P0) (27,28). The AR staining localized to the cytoplasm of Leydig cells in mouse testis at E16 and P0 (27), which is in contrast to expression in the adult where staining was prominent in the nucleus (28). The epithelium of the mesonephros-derived tissues, including the rete testis and the epididymis, exhibited a higher capacity to
express the AR than did the rest of the testicular tissue (26). Therefore, we determined whether ARA70 is localized with the AR during early embryogenesis, when estrogen is synthesized in the fetal testis. Mouse IgG was used as a negative control for staining (A, D and G).

Fig. 8, C and F, show that ARA70 was present in the interstitial cells of E16 and P0 mouse testis, similar to the AR expression (B and E). The interstitial space contains Leydig cells located between the seminiferous tubules. ARA70 can also be detected in the vas deferens and epididymis, similar to the expression of the AR at E16 and P0 (data not shown). Furthermore, ARA70 was present in both the cytoplasm and the nuclei of the testicular Leydig cells of 7 weeks old mice (I). Whereas the AR is present in the cytoplasm during E16 and P0, and localized in the Leydig cell nuclei at 7 weeks (H). These data indicate that ARA70 expression begins in the Leydig cell, at the time of testosterone and E2 synthesis, during embryogenesis.

**DISCUSSION**

Androgen insensitivity syndrome is characterized by a spectrum of deficient male virilization or undermasculinization including testicular feminization (29). It is known that if androgen action is slightly less than optimal, there will be undervirilization or inefficient spermatogenesis (29). While mutation of the AR is often found in PAIS patients, certain mutations associated with PAIS respond normally to androgen (4). The first mutation analysed in this study, AR(S703G), was identified in a patient diagnosed with male pseudohermaphroditism which is associated with PAIS (3). The other mutation we studied, AR(E709K), is associated with Reifenstein syndrome (13). Reifenstein syndrome is a less severe variant of X-linked PAIS in which affected individuals present as phenotypic males with hypospadias, inadequate pubertal virilization, gynecomastia, and infertility (30).
Figs. 1 to 4 show that both of these PAIS mutants respond to androgen normally, but have lost the ARA70-dependent response to E2. Although E2 is a non-specific ligand for the wtAR, certain coregulators, such as ARA70, or SRC-1, can enhance wtAR transactivation in response to E2 (13-16). E2 and DHT have similar structures except that E2 has a phenolic A-ring. It is therefore possible that while the wtAR may be able to bind to E2, E2 may not be well oriented within the wtAR-LBD pocket leading to weaker binding. Therefore, E2 may easily dissociate resulting in marginal activation of the wtAR. The recruitment of ARA70 or other selective coregulators to the wtAR, however, may change the conformation of the wtAR so that E2 can be trapped within the wtAR-LBD pocket allowing E2 to more effectively activate wtAR transactivation. In terms of mutants, the conformation of E2 bound AR mutant may not be similar to that of E2 bound wtAR. That difference in the conformation may influence on the receptor-interface providing for the coregulator recruitment.

Our data in Figs. 5 and 6 show that ARA70 helps E2 competitively bind to the wtAR and subsequently retards E2 dissociation from the wtAR binding pocket. This phenomenon may be important during the prenatal period (7) where high maternal estrogen levels may initiate estrogen signaling via the wtAR in the presence of coregulators, such as ARA70. For example, one report found that plasma concentrations of testosterone and E2 in mothers delivering male babies were 1.2±0.4 and 4.5±0.7 ng/ml, respectively (31), and these sex hormone levels may influence the balance between androgens and estrogens in the male fetus. Studies of 20 fetal rat testis samples collected during genital tract differentiation (gestation) also reported that there is a negligible level of circulating DHT in fetal rat plasma (32). In addition, male and female rat fetuses had similar plasma levels of DHT and E2, although male fetuses did have higher levels of testosterone (32). High aromatase activity during the last days of fetal life may also convert testosterone to E2 in male fetuses (32,33), further altering the
balance of these sex hormones. Therefore, while male sexual differentiation and reproductive organ development are critically dependent on androgen-AR signaling, it is possible that estrogen-AR-coregulator signaling may influence androgen action in conditions where DHT levels are negligible and testosterone is readily aromatized to estrogen. The loss of the E2-AR-coregulator pathway may partly disrupt the balance between AR and ER signaling during critical stages in male reproductive system development. Fig. 4B demonstrates that the S703G and E709K PAIS mutants cannot functionally interact with ARA70 in response to E2. Therefore, decreased total receptor activity in AR(S703G) and AR(E709K) PAIS patients may tip the balance in favor of ER signaling, resulting in undermasculinization.

Our in vivo data in Fig. 8 shows that ARA70 and the AR are both present in the interstitial cells of the mouse E16 testis. Primitive sex cords mature to form testis cords and the rete testis at 13 days post coitum (34). By 15 days, the vas deferens, epididymis and seminal vesicles arise from the Wolffian duct and the testis develops prominent sex cords, each of which become a seminiferous tubule (34). At this point, the cord contains uniform but undifferentiated cells, which are precursors of spermatogenic or Sertoli cells. As the testis continues to develop, mesenchymal cells between the testis cords become Leydig cells. Leydig cells are responsible for the synthesis and secretion of testosterone, the primary hormone of the testis and a critical regulator of spermatogenesis. Leydig cells also produce 5α-reduced androgens, such as DHT and 3α-diol. E2 can be synthesized through the aromatization of testosterone by aromatase in the Sertoli cells and diffuse back to the Leydig cells (33,34). Conversion of testosterone to estrogen also occurred in the Leydig cells. Testicular testosterone and its derivatives then induce masculine extragonadal differentiation. The abundance of ARA70 in testicular interstitial cells, at the time of E2 synthesis, supports our in vitro data showing the modulation of ARA70 on the AR-E2 binding. Moreover, this could imply that ARA70 impact the development of
male structures via not only androgen-AR-ARA70 pathway, but also E2-AR-ARA70 pathway, to stimulate the development of genital organs, such as the vas deferens, epididymis, and seminal vesicle, and the masculinization of the external genitalia.

Our data therefore provide one possible molecular basis for a subset of PAIS where mutation does not abolish androgen-AR signaling. Recent studies reported that ARA70 is not mutated in an analysis of 27 androgen insensitivity syndrome patients (35). This finding excludes the possibility of ARA70 mutation in PAIS, and supports our assertion that the loss of AR mutant functional interaction with ARA70 due to helix 3 mutations may affect a subset of PAIS patients where androgen response is normal.

In summary, our data provide one of the potential molecular causes to explain the development of PAIS in a subset of patients with AR mutants that respond normally to androgen. Moreover, our results have partially elucidated how ARA70 enhances the activation of the wtAR in response to E2. These findings suggest that certain coregulators may have an important role in the regulation of male reproductive system development by E2 induction of the AR transactivation.

Acknowledgments
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REFERENCES


**FIGURE LEGENDS**

**FIG. 1.** **ARA70 enhances the E2-mediated transactivation of wtAR, but not PAIS mutant AR(S703G) or AR(E709K).** 

A, A schematic representation of the helix 3 regions of AR, ER, GR, and PR showing the locations of the S703G, N705S, L707R, and E709K mutations (3,4). 

B, shows DHT-dependent, but not E2-dependent, transactivation of AR(S703G) or AR(E709K) PAIS mutant in the presence of ARA70. AR-negative DU145 cells were co-transfected with MMTV(AR)-CAT reporter (3.5 µg) and pSG5 wtAR or various AR mutants (1.5 µg) in the presence or absence of pSG5 ARA70 (4.5 µg) under either 10 nM DHT (lanes 2-3) or 10 nM E2 (lanes 4-5) treatment. Equal amounts of pSG5 vector (4.5 µg) were used to substitute for pSG5-ARA70 in the wtAR or mutant AR alone transfection. CAT activity was normalized according to β-galactosidase activity and fold CAT activity is expressed based on the induction fold relative to ethanol treatment (set as 1 fold). Data represent the mean ± S.D. of three individual experiments. *, p < 0.05, significant activation of AR mutant transactivation by DHT compared with vehicle treatment by Student's t test. **, p < 0.05, significant increase in DHT-induced wtAR or AR mutant transactivation by ARA70 compared with wtAR or AR mutant alone set.

**FIG. 2.** **E2 binds to the wtAR, AR(S703G), or AR(E709K).** 

[^H]-R1881 bound (cpm) to the AR in a competitive ligand binding assay using[^H]-R1881 in the presence or absence of unlabeled R1881 or unlabeled E2 was shown. AR-negative H1299 cells were transiently transfected with the wtAR or the AR mutants with 20 nM[^H]-R1881 in the presence or absence of a 100-fold excess of unlabeled R1881 or unlabeled E2 (as a...
The competitive binding ability of R1881 or E2 to the AR was evaluated by the ability of unlabeled R1881 or unlabeled E2 to compete with a saturating amount of [3H]-R1881. The [3H] count was determined using a scintillation counter. The values are the means of three individual assays.

**Fig. 3.** E2 promotes nuclear translocation of the wtAR, AR(S703G), and AR(E709K). A and B show the translocation of AR(S703G) and AR(E709K) PAIS mutants from the cytoplasm to the nucleus after DHT (A) and E2 (B) treatment. COS-1 cells were transiently transfected with the pSG5-wtAR or with various AR mutants (2 µg) in the presence or absence of 10 nM DHT or 10 nM E2. The wtAR or AR mutants were detected using an anti-AR antibody (NH27), and visualized by rodamine-fluorescence. A, Cells were photographed under confocal microscopy at a magnification of x400, in series of 8 stacks (the entire "Z" plane of the cells) to demonstrate the translocation of AR signal from the cytoplasm to the nucleus after 10 nM DHT treatment. B, Cells were photographed under confocal microscopy at a magnification of x400, at the mid-plane of the nucleus to determine the AR signal in the nucleus of the cell after 10 nM E2 treatment. The location of the cell nuclei was confirmed by staining using DAPI. C, Quantitative analysis of cytoplasmic and/or nuclear staining of wtAR and AR mutants. A total population of 50 transfected cells (n=50), under E2 treatment, was assayed in each of three different experiments. Three distinct cell populations were found: (1) AR staining in the cytoplasm (C), (2) AR staining homogenously distributed through out both the cytoplasm and the nucleus (C+N), and (3) AR staining in the nucleus (N). *, p < 0.05, significant translocation of AR signal by E2 compared with vehicle treatment by Student's t test.
**FIG. 4.** **ARA70 interacts with the wtAR, but not with AR(S703G) and AR(E709K), in the presence of E2.** A, Co-immunoprecipitation of exogenously expressed wtAR and ARA70 in COS-1. The wtAR interacts with ARA70 by co-immunoprecipitation in an agonist-enhanced manner. COS-1 cells were transfected with pSG5 vector (10 µg) or pSG5AR (2.5 µg) and pSG5ARA70 (7.5 µg) using Superfect (Qiagen). After transfection, the cells were treated with vehicle, 10 nM DHT, or 10 nM E2. The wtAR complexes were immunoprecipitated using NH27 and protein A/G beads. The membrane was blotted with antibody NH27 for wtAR and the lower portion was blotted with antibody CC70 for ARA70. B, The differential mammalian two-hybrid interaction of AR(S703G) or AR(E709K) and ARA70 under E2 vs. DHT treatments in DU145 cells. DU145 cells were transiently transfected with a DNA mixture of pG5CAT (3.5 µg), pCMV-β-gal (1 µg) and an equal amount of fusion-plasmids (3 µg). The two-hybrid interaction was standardized by the measurement of β-gal activity. CAT activities were expressed as fold stimulation above untreated Gal4 (lane 1) or untreated, DHT, or E2 treated Gal4/VP16 (lanes 2 to 4) samples. Data presented represent the mean ± S.D. of three individual assays.

**FIG. 5.** **ARA70 influences the competitive-binding of E2 to the wtAR, but not AR(E709K), at unsaturated androgen levels.** A, To determine the effect of ARA70 on the binding of R1881 to the wtAR, transfected cells were incubated with medium containing 0.1 to 20 nM [3H]-R1881 in the presence or absence of a 100-fold excess of unlabeled R1881. B Upper, Schematic shows the experimental design of B Lower to demonstrate the increased formation of E2-AR-ARA70 at the unsaturated androgen level. B Lower, [3H]-R1881 bound (cpm) to the wtAR or mutant in the presence (lanes 2 to 7) or absence (lane 1) of unlabeled E2 competitor. DU145 cells transfected with wtAR or AR(E709K) (1.5 µg), with or without ARA70 (4.5 µg), were labeled with 1 pM [3H]-
R1881 (unsaturated concentration) and then competed with 0.01, 0.1, 1, 10, 100, and 1000 nM unlabeled E2. Equal amounts of pSG5 vector (4.5 µg) were used to substitute for pSG5-ARA70 (4.5 µg) in the wtAR or AR(E709K) (1.5 µg) alone transfections. Lane 1 demonstrates the comparison of [³H]-R1881 bound to the wtAR or AR(E709K) alone vs. wtAR or AR(E709K) cotransfected with ARA70 in the absence of unlabeled E2. Lanes 2 to 7 demonstrate [³H]-R1881 bound to the wtAR or AR(E709K) alone vs. wtAR or AR(E709K) cotransfected with ARA70 in the presence of unlabeled E2. The values are the mean ± S.D. of three individual assays. The AR-E2 binding was evaluated by the competition of unlabeled E2 to [³H]-R1881 [the AR-E2 binding = [³H]-R1881 count in the absence of unlabeled E2 - [³H]-R1881 count in the presence of unlabeled E2]. *, p<0.05, significant increase in unlabeled E2 competition for [³H]-R1881 binding to the wtAR in the presence of ARA70 compared with the wtAR alone which set as a control.

**FIG. 6.** ARA70 retards the dissociation of E2 from the wtAR, but not from AR(E709K). [³H]-E2 bound (cpm) to the wtAR or mutant AR, AR(E709K), alone is compared to wtAR or mutant AR, AR(E709K), cotransfection with ARA70 in the presence of unlabeled E2. The wtAR (A and B, C and D) or AR(E709K) (E and F) (1.5 µg), with or without ARA70 (4.5 µg), were transiently expressed in DU145 cells. Equal amounts of pSG5 vector (4.5 µg) were used to substitute for pSG5-ARA70 in the wtAR or AR(E709K) transfections without ARA70. Transfected cells were treated with 0.1 nM of [³H]-R1881 (A and B) or [³H]-E2 (C and D, E and F), in the absence or presence of 100-fold excess unlabeled R1881 (A and B) or E2 (C and D, E and F), for 2 h at 37°C. Specific binding activity was determined as the difference in counts between those in the absence and those in the presence of 100-fold excess unlabeled R1881 or E2. After 2 h,
the medium was changed to that with or without a 10,000-fold molar excess of unlabeled R1881 (B) or E2 (D,F), at 37°C. The cells were harvested at the indicated times, and the radioactivity was measured. The values are the means ± S.D. of three individual assays. *, p < 0.05, significant decrease in [3H]-E2 dissociation from the wtAR in the presence of ARA70 compared with the wtAR alone.

**Fig. 7.** ARA70 does not influence the association of E2 with the wtAR and AR(E709K). [3H]-E2 bound (cpm) to the wtAR (A) or AR(E709K) (B) with or without ARA70. DU145 cells were transiently transfected with wtAR or AR(E709k) (1.5 µg), with or without ARA70 (4.5 µg). An equal amount of pSG5 vector (4.5 µg) was used to substitute for pSG5-ARA70 in the wtAR or AR(E709K) alone transfection. Cells were incubated in serum-free medium containing 0.1 nM [3H]-E2 at 37°C, and harvested to measure the binding at the indicated times. At each time point, the selected cell plates were extensively washed with cold 1X PBS. The samples were blocked by 100-fold excess of unlabeled E2. The values are the means ± S.D. of three individual assays.

**Fig. 8.** Immunohistochemical analysis of AR and ARA70 expression in testicular Leydig cells. Figures show the interstitial space between the seminiferous tubules. Wild type mouse testis at embryonic day 16 (E16) (A-C), Postpartum day 0 (D-F), and Postpartum day 7 weeks (G-I) were stained with mouse IgG (negative control) (A, D and G), mouse AR (G122-77, Pharmedgen International) (B, E and H), or mouse ARA70 (CC70) (C, F and I) antibodies. A peroxidase Immunodetection Kit (PK-2200, Vector Laboratories) was used, and sections were counterstained with hematoxylin. Data are representative of at least three independent experiments. Leydig cell nuclei are indicated by arrows. Magnification, x400.
A. Partial androgen insensitivity

Complete androgen insensitivity

<table>
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<td>hER</td>
<td>ASMMGLLTNLADKELVEMINNAK</td>
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<td>hGR</td>
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</tr>
<tr>
<td>hPR</td>
<td>SSLTSLNLQGERQLLSVVKWSK</td>
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B. MMTV(ARE)-CAT/ DU145

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<tr>
<th>Condition</th>
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<td>![Bar Chart]</td>
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<tbody>
<tr>
<td>PSG5-ARA70</td>
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<td>-</td>
</tr>
<tr>
<td>ETOH</td>
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<td>-</td>
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</tr>
<tr>
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<td>+</td>
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</tr>
<tr>
<td>10 nM E2</td>
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<td>-</td>
<td>+</td>
</tr>
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</table>

Figure 1. Thin et al.
Figure 2. Thin et al.

[Diagram showing the binding of [³H]-R1881 to different AR mutants and the effect of R1881 and E2 competition.]

<table>
<thead>
<tr>
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</tr>
<tr>
<td>E709K</td>
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</tr>
</tbody>
</table>
Figure 3A. Thin et al.
Figure 3(Band C). Thin et al.

B. DAPI 10 nM E2

C. COS-1
3X(N=50)

AR staining cells

Cellular compartment

wtAR  wtAR S703G  wtAR E709K  wtAR S703G E709K
Figure 4. Thin et al.
Figure 5. Thin et al.

A.

<table>
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<th>Binding Affinity (Kd) of R1881 (nM)</th>
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<tr>
<td>AR + ARA70</td>
</tr>
<tr>
<td>1.82 ± 1.21</td>
</tr>
</tbody>
</table>

B.

Unsaturated androgen level

Androgen receptor response

Androgen level

10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} (M)

AR/ Control

AR + ARA70

AR(E709K)

AR(E709K) + ARA70

DU145

1 pM[^3]H-R1881

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<th>DU145</th>
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<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 6(A and B). Thin et al.

A.

B.
Figure 6(C to F). Thin et al.

C.  

D.  

E.  

F.
Figure 7. Thin et al.

A.

B.
Figure 8. Thin et al.
Mutations in the helix 3 region of the androgen receptor abrogate ARA70 promotion of 17b-estradiol-induced androgen receptor transactivation

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