The orphan receptor ERRα interferes with steroid signaling

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ABSTRACT

The estrogen receptor-related receptor α (ERRα) is an orphan member of the nuclear receptor super-family that has been shown to interfere with the estrogen-signaling pathway. In this report, we demonstrate that ERRα also cross-talks with signaling driven by other steroid hormones. Treatment of human prostatic cells with a specific ERRα inverse agonist reduces the expression of several androgen-responsive genes, in a manner that does not involve perturbation of androgen receptor expression or activity. Furthermore, ERRα activates the expression of androgen response elements (ARE)-containing promoters, such as that of the prostate cancer marker PSA, in an ARE-dependent manner. In addition, promoters containing a steroid response element can be activated by all members of the ERR orphan receptor subfamily, and this, even in the presence of antisteroid compounds.

INTRODUCTION

The estrogen receptor-related receptor α (ERRα) is a member of the nuclear receptor superfamily, that, together with its paralog ERRβ, has been identified due to its high level of sequence identity with the estrogen receptor α(ERα) (1). A third member of the subfamily, ERRγ, has also been isolated more recently (2). These three receptors are referred to as 'orphan', since their transcriptional activities do not require the addition of an identified physiological ligand (3). Crystallographic studies have shown that apo-ERRα and apo-ERRγ spontaneously adopt an active conformation, indicating that they can activate transcription in a constitutive manner (4,5). A specific, synthetic inverse agonist (XCT790) of

ERRα has however been identified that deactivates the receptor (6) and induces its proteasome-dependent degradation (7), thus facilitating the study of its activities, at least in cell culture.

ERRβ has been shown to be involved in the formation of the placenta and to regulate the maturation of primordial germ cells (8,9), whereas ERRγ is essential in the oxidative metabolism of the postnatal heart (10). Published data from various laboratories have shown that ERRα plays a significant role in the regulation of energy homeostasis. Indeed, ERRα is highly expressed in tissues with high energy demands such as the heart or skeletal muscle (11–13). ERRαKO mice display a resistance to diet-induced obesity, altered fat metabolism and adsorption (14–16), fail to maintain body temperature when exposed to cold (17) and respond in an altered manner to cardiac pressure overload (18). These activities are thought to be exerted mainly in cooperation with the PPARγ coactivator 1-α (PGC1-α), which is instrumental in many metabolical processes (13,19–20). However, ERRα is also expressed in many other tissues, in which its physiological roles has not been determined (11). The receptor has been proposed to act as a modulator of bone mass (21), and its high expression in ovarian, colon and breast cancers correlates with poor prognosis (22–26) although the functions of ERRα in cancer have not been investigated.

The structural proximity between ERRs and ERs, in particular in the DNA-binding domain (DBD) has led to hypothesis that the ERRs could interfere with the estrogen pathway (27). Indeed it has been shown that, at least in certain cellular and promoter contexts, some ERR response elements (ERREs) could transduce an estrogen signal (28–30), as illustrated by the case of the osteopontin gene promoter (31,32). In addition, such genes as lactoferrin or the breast cancer marker pS2, can be coregulated by both ERRα and ERα, through neighboring sites on their promoter (33,34). Furthermore, ERRα has been proposed to regulate the

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expression of aromatase (35), the rate-limiting enzyme in estrogen biosynthesis, and the expression of ERRα itself is reportedly induced by estrogens in the mouse uterus (36). Phylogenetical studies have grouped the ERRs in nuclear receptor subfamily 3, not only together with the ERs but also with the other steroid receptors [StRs, namely androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)] (37). This raises the hypo thesis that ERRs can also interfere with steroid signaling.

In this report, we show that the expression of androgen-responsive genes can be down-regulated by the ERRα-specific inverse agonist XCT790 in LNCaP (androgen-dependent human prostate cancer) cells. Importantly this effect of XCT790 is not exerted through a modulation of AR expression or activity. ERRα stimulates the activity of androgen-responsive element (ARE)-containing promoters, including that of PSA, a marker of prostate cancers. Other steroid-response elements (StRE)-containing promoters can also be activated by ERRs even in the presence of antisteroid compounds. Our results thus document a new level of interferences of ERRs in hormonal pathway.

MATERIALS AND METHODS

Plasmids

ERRα mutants have been described elsewhere (38). For construction of LEF-RE-Luc reporter plasmid, an oligo-nucleotide (sequence: 5′-GATCCGCACCCTTTGAAGC TCA-3′) encompassing the cognate sequence was cloned as a trimer in plasmid pGL3-promoter (Promega, Charbonnières, France) and the construct was verified by sequencing. The following plasmids were gifts: PSA promoter derivatives (HW Chen, UC Davis; described in ref. 39), scARE2.1-Luc (F Claessens, Leuven University), DR1-Luc (H Escriva-Garcia, Banyuls-sur-Mer), PGC1-α (A Kralli, Scripps Institute, San Diego), ARR2PB-Luc (RJ Matusik, Vanderbilt University; described in ref. 40), SRC1 (MG Parker, Imperial College, London), GRIP1, GalAR-AF1, GalAR-LBD (MR Stallcup, USC Los Angeles; ref 41), pS2-Luc (V. Cavaillès, Montpellier), PerRE-Luc (G. Triqueneaux, ENS Lyon), MMTV-Luc, GR, DNA-binding deficient GR mutant (GRdim) (42), AR, ARE-Luc and GRE-Luc (M. Resche-Rigon, Prostrakan, Romainville). ARE-Luc contains a trimer of the ARE from the rat probasin promoter (sequence: 5′-AG CTTAATAGGTTCTTGGAGTACTTTACGTCGA-3′, consensus ARE underlined; ref. 43) cloned in front of the *collagenase* promoter, scARE2.1 contains four copies of an ARE (sequence: 5′-GGCTCTTTCAGTTCT-3′, ARE underlined; ref. 44) from the secretory component gene cloned in front of the minimal *tk* promoter). GRE-Luc contains an hexamer of the glucocorticoid response elements (GRE) from the metallothionein promoter (sequence: 5′-CGGTACAAATGTTCTGGGCC-3′, consensus GRE underlined; ref. 45) cloned in front of the minimal early *SV40* promoter.

Cells and transfections

LNCaP cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 10 mM Hepes and 1 mM sodium pyruvate. HeLa and PC3 cells were cultured in DMEM medium supplemented with 10% fetal calf serum. For transient transfections 10^5 cells were seeded in 24-well plates and transfected using 3 µl of ExGen 500 (Euromedex, Souffelweyersheim, France), 50 ng Luciferase reporter plasmid. CMV-βGal plasmid (50 ng) was added to normalize transfection efficiency and pSG5 plasmid was added as a carrier up to 500 ng. Cells were lyzed 48 h after transfection and reporter activities were determined, using standard methods. All transfections were performed in triplicate. Dexamethasone (Dex), RU486, dihydrotestosterone (DHT), trichostatin A (TSA), flutamide and XCT790 were purchased from Sigma Aldrich (St Quentin Falavier, France) and resuspended in DMSO.

The sequences of the siRNAs used to target ERRα are as follow: sense 5′-GGCAGAAACCUAUCUCAGGUU-3′, antisense 5′-CCUGAGAUAGGUUUCUGCCUC-3′. The siRNA control was provided by Dharmacon (si*CONTROL* Non-Targeting siRNA #1). Annealed siRNAs were transfected with Lipofectamine 2000 (Invitrogen, Cergy Pontoise, France) into LNCaP cells in 6-well plates first for 48 h. Cells were transfected a second time with siRNAs and then treated for 48 h with XCT790. RNA extraction was performed and mRNA levels for the indicated genes were determined by quantitative PCR (QPCR) and normalized to the 36B4 mRNA level. Parallel cell extracts were prepared for immunoblots.

Expression analysis

RNAs were purified using Guanidinium thiocyanate/phenol/chloroform extraction. Two micrograms total RNA were DNaseI-digested and retrotranscribed in a final volume of 20 µl using SuperScript II retrotranscription kit (InVitrogen, Cergy-Pontoise, France) under the conditions recommended by the supplier. QPCR were performed using the Sybr Green Jump Start kit (Sigma Aldrich) in duplicate on a ABI apparatus using standard PCR cycle. Specific primers for *MAK* and *KLK2* were from refs (46) and (47), respectively.

Primers used in this study:

36B4 5′-GTCACTGTGCCAGCCCAGAA-3′ and 5′-T CAATGGTGCCCCTGGAGAT-3′; *AR* 5′-CCTGGCTT CCGCAACTTACAC-3′ and 5′-GGACTTGTGCATG CGGTACTCA-3′; *ERRα* 5′-CAAGCGCCTCTGCCTG GTCT-3′ and 5′-ACTCGATGCTCCCCTGGATG-3′; *KLK2* 5′-CATCCAGTCTCGGATTG-3′ and 5′-CTCA TATTGTAGAGCGGGT-3′; *MAK* 5′-GGTCACAGC CACCATACACTGA-3′ and 5′-ACTTCCAACAGCCC ACACATC-3′; *MAO-A* 5′-ACCAAGCCAGATGGGT CA-3′ and 5′-TTTCCGGGCAAGAATGAA-3′; *MCAD* 5′-ATTGGTGACGGAGCTGGTTT-3′ and 5′-TCCAA CAGCACCAGCAGCTA-3′; *NKX3.1* 5′-GCCGAGAC GCTGGCAGA-3′ and 5′-CAGATAAGACCCCAAGT GCCTTT-3′; *PGC1-α* 5′-TGGTGCAGTGACCAATCA GAAA-3′ and 5′-GGACTGCTAGCAAGTTTGCCTC-3′; *PSA* 5′-ACCAGAGGAGTTCTTGACCCCAAA-3′

and 5′-CCCCAGAATCACCCGAGCAG-3′; *RIP140* 5′-C AGTTCCACTCAGCCCAGCA-3′ and 5′-ACAGCCA ACGTGCTCAGGGA-3′.

Western blot

Cells were lyszed in RIPA buffer (50 mM Tris, pH 7.5; 150 mM NaCl; EDTA 5 mM; 0.5% Nonidet P-40; 0.1% sodium deoxycholate; 0.1% SDS and a cocktail of protease inhibitors) then centrifuged 15 min at 13 200 rpm. After 10 min heating in Laemmli's buffer, proteins (30 µg) were resolved on 8% SDS–polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane (Amersham Biosciences, Orsay, France) and blocked by incubation at room temperature for 1 h in TBS–Tween 0.1% containing 5% nonfat dry milk. Blots were probed with various Abs (AR, ERRα and actin) and developed using an enhanced chemiluminescence detection system (ECL kit, Amersham Biosciences) with appropriate specific peroxydase conjugated Abs. Anti-AR and antiactin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma Aldrich, respectively. Anti-ERRα has raised in rabbit, using a KLH-coupled peptide mapping a region conserved between mouse and human ERRα, but divergent in other ERR subfamily member.

ChIP

ChIP assays were performed as described previously (48). Briefly, LNCaP cells were grown in phenol red-free RPMI 1640 supplemented with 10% dextran/charcoal-stripped serum in 150 mm dishes for 3 days, and then treated with or without 10 nM DHT for 2 h. A small portion of the crosslinked, sheared chromatin solution was saved as input DNA, and the remainder was precleared by incubating with 20 µl of preblocked protein A agarose/Salmon Sperm DNA (Upstate Biotech, Chandlers Ford, UK) for 2 h. The precleared chromatin was then immunoprecipitated with 2 µg of anti-AR antibody (H-280, Santa Cruz Biotechnology) or 2 µg of anti-ERRα antibody (R&D systems, Minneapolis, MN, USA) with rotation overnight at 4°C. The next day, immunoprecipitated DNAs were recovered by incubated with 20 µl of preblocked protein A agarose/Salmon Sperm DNA (Upstate Biotech) for 2 h. The immune complexes were eluted from beads with an elution buffer (1% SDS and 0.1 M NaHCO3). Crosslinking was reverse overnight at 65°C and DNAs were purified using QIAquick column (Qiagen, Courtabœuf, France). Real-time PCR were performed using 2 µl DNA in duplicate on a ABI apparatus using standard PCR cycles. Primers used in this study have been previously described (49). Identical results were obtained when using our own anti-ERRα antibody.

RESULTS

To determine whether ERRα interacts with steroid signaling, we first focus on androgen signaling in LNCaP cells. These cells were treated with the specific ERRα inverse agonist XCT790 for 24 h. As judged by QPCR, this compound reduced the expression of several androgen-responsive genes [*PSA/KLK3*, *NKX3.1*, *KLK2* and *MAK1*; (46,50–52)] nearly as efficiently as did flutamide, a specific antiandrogen (Figure 1A). This effect is not due to a general aspecific inhibitory effect of XCT790 since the expression of PGC1-α was not modified. As controls, the expression of ERRα validated target genes, such as MAO-A, MCAD and RIP140 (6,14,53) were also downregulated by XCT790 in LNCaP cells (Figure 1B). No synergy was observed between flutamide and XCT790. The latter acted in a time- and dose-dependent manner (Figure 1C and D, respectively) with a half-maximum efficiency on both PSA and MCAD expression of 5×10^{-6} M, a concentration which we used hereafter. TSA [a histone deacetylase (HDAC) inhibitor] induced in an overexpression of PSA (Figure 1E) as reported (54). Upon cotreatment with TSA and XCT790, PSA expression displayed an intermediate level, suggesting that the two phenomena are independent. XCT790 is thus unlikely to induce HDAC recruitment to the PSA promoter. To assess the specificity of XCT790, LNCaP cells were treated with an siRNA directed against ERRα (siE). This treatment had no effect on PSA expression, although it efficiently down-regulated the expression of ERRα mRNA and protein (Figure 1F), suggesting that other factors could compensate for the absence of ERRα. However, XCT790 had no effect on PSA expression when used on siE-treated cells, indicating that the drug absolutely required ERRα to inhibit PSA expression.

We next address whether ERRα could transcriptionally act on the PSA promoter. To this end, we cotransfected HeLa cells that were chosen because they do not express PSA. Full-length PSA promoter was activated by ERRα and by AR (the latter, but not the former, requiring DHT, as expected) (Figure 2A). A deletion construct in which the 5′ enhancer was absent displayed a reduced response to AR as published (46), and did not react at all to ERRα, suggesting that the deleted 5′ region could be involved in the response to both AR and ERRα. As expected, activation of the PSA promoter by ERRα was inhibited by XCT790 treatment (Figure 2B). To determine whether ERRα bound to the endogenous PSA gene, we performed chromatin immunoprecipitation (ChIP) experiments followed by QPCR targeting specific regions of the PSA promoter (Figure 2C). ERRα was recruited to the enhancer (Enh) region to a low level, but not to the intermediate (E–P) or promoter (P) regions. Interestingly XCT790, but not DHT, significantly enhanced the recruitment of the receptor specifically to the Enh region. Altogether, this indicates that ERRα transcriptionally activates the expression of PSA by binding to its Enh region. XCT790 enhances the binding of the receptor and suppresses its activity.

The above results suggest that ERRα could activate transcription through AREs, several copies of which are present in the PSA Enh region. To test this hypothesis, we used other plasmid constructions in which AREs were present in various configurations. A composite rat probasin promoter derivative (ARR2PB; 40) was activated by ERRα in a dose-dependent manner without exogenously added DHT (Figure 3A). In contrast to ERRα, AR achieved a maximal activation level only in the presence of DHT. ERRα also
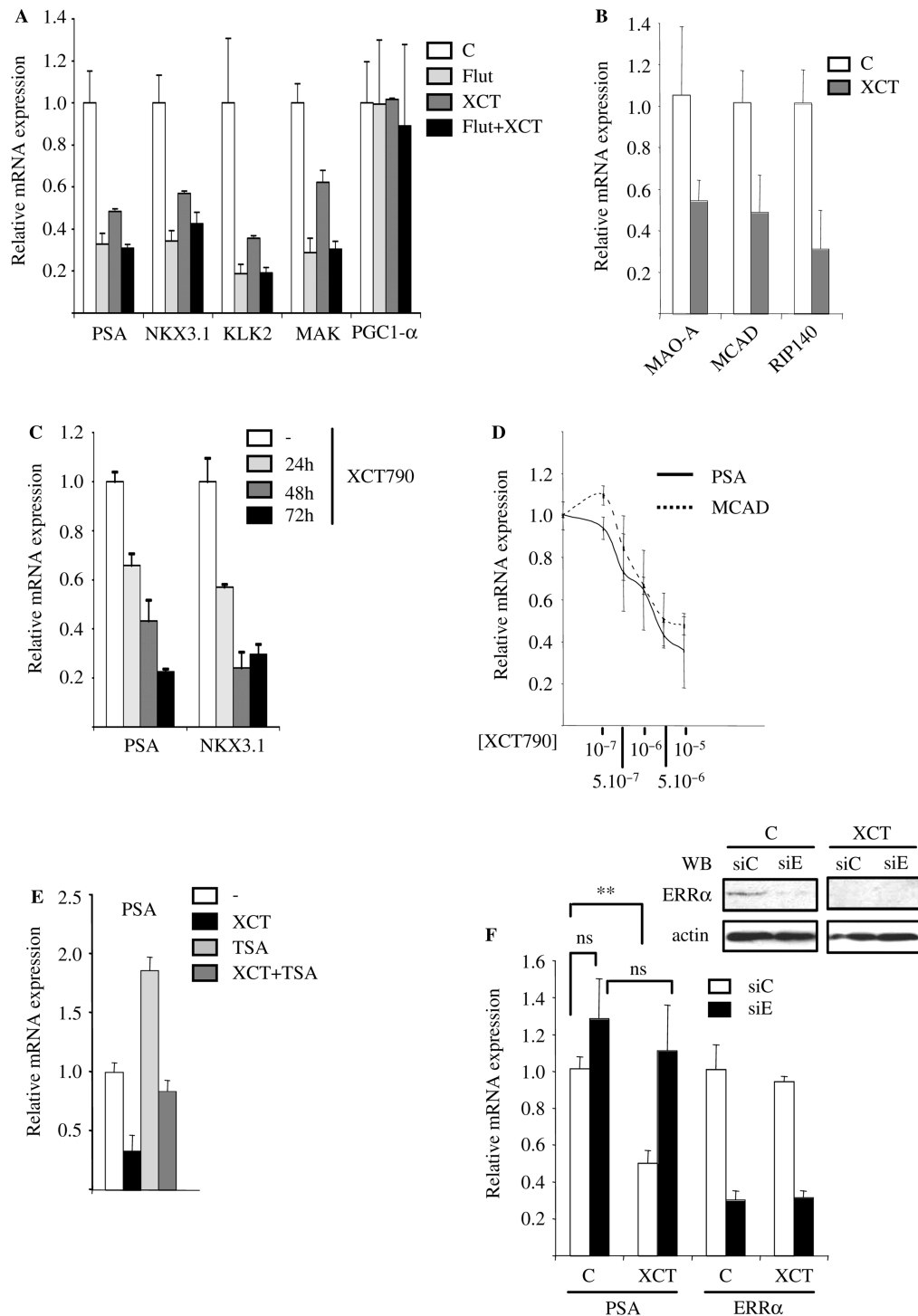
Figure 1. XCT790 regulates the expression of androgen-responsive genes in LNCaP cells in an ERRα-dependent manner. (**A**) Cells were treated for 24 h with 5×10^{-6} M XCT790, 10^{-6} M flutamide or a combination of both, as indicated. RNA were extracted, retrotranscribed and analyzed by QPCR. Expressions were normalized against 36B4 level and expressed relative to untreated control (**C**) arbitrarily assigned to 1. Results represent the average of a typical triplicate experiment with standard deviation as error bar. Experiments were performed at least three times. (**B**) Same as in (A) using XCT790 treatment only. (**C**) Cells were treated with 5×10^{-6} M XCT790 for the indicated time. PSA and NKX3.1 expression were analyzed as in (A). (**D**) Cells were treated with the indicated XCT790 concentrations for 24 h. PSA and MCAD mRNA expression were analyzed as in (A). Results are depicted with a logarithmic x scale. (**E**) Cells were treated with XCT790, TSA (125 ng/ml) or both for 72 h, with medium renewal every 24 h. PSA was analyzed as in (A). (**F**) Cells in 6-well plate (triplicate) were transfected with 25 pmol/well of ERRα-directed (siE) or unrelated control (siC) siRNA as described in Materials and methods section and treated or not with XCT790 for 48 h. PSA and ERRα mRNA expression were analyzed as in (A). Protein levels were determined by immunoblots from cell extracts of a fourth siRNA-transfected well. Protein results are from a single experiment that is representative of three independent ones. mRNA results are the average of three experiments with error bars indicating standard deviation. ANOVA tests were performed to determine statistical significance; ns: nonsignificant, **$P < 0.01$.
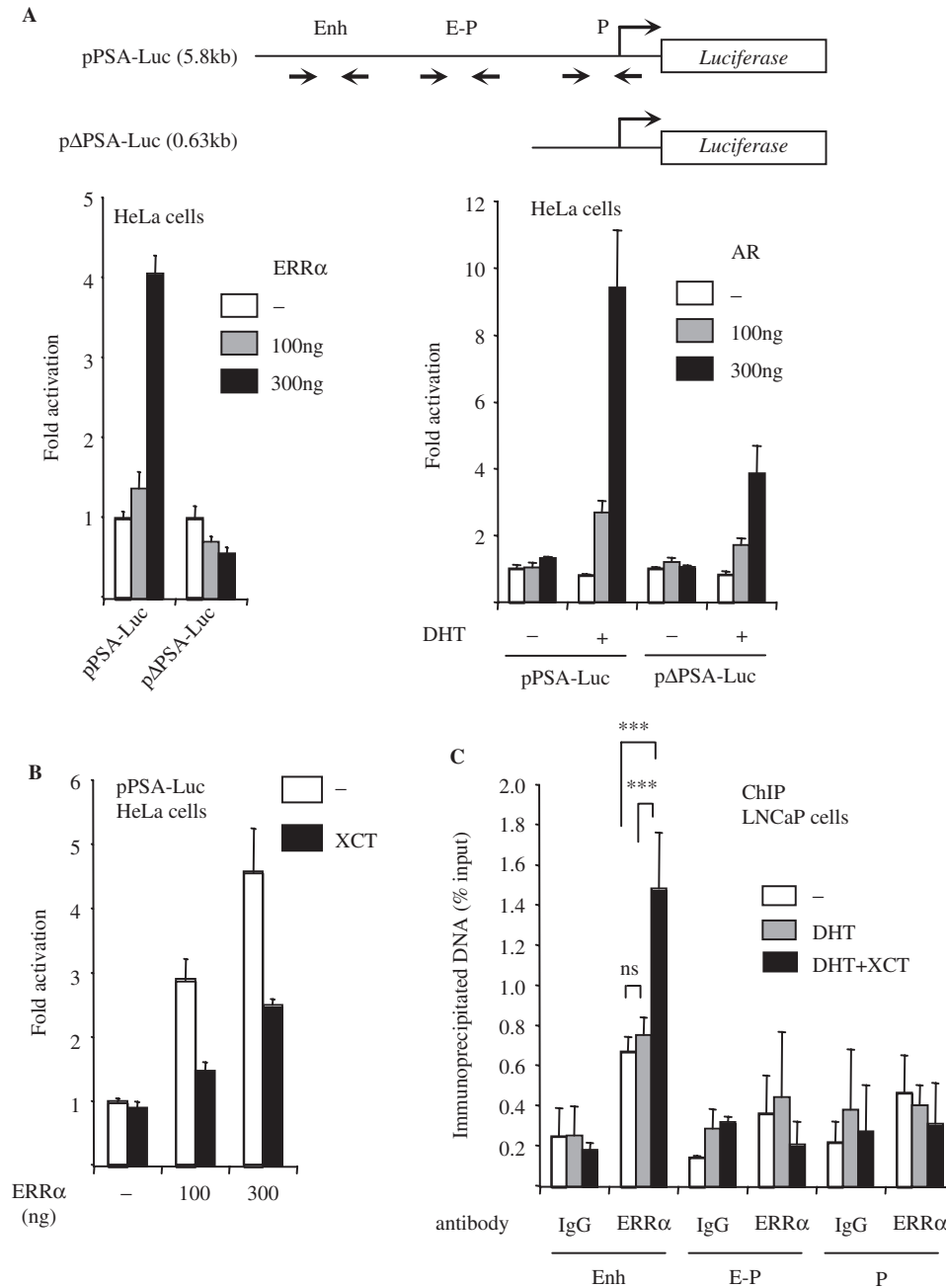
Figure 2. ERRα activates the PSA gene promoter. (**A**) A schematic representation of the used PSA reporter constructs is depicted at the top, in which the regions amplified by QPCR after ChIP are indicated. HeLa cells were transiently transfected with the indicated reporter constructs together with the varying amounts of ERRα (left panel) or AR (right panel) encoding plasmids. CMVβGal was added as a transfection efficiency control. Where indicated DHT (10^{-8} M) was added to the culture medium. Luciferase activity was determined 48 h after transfection and normalized against βGal activity. Results are expressed as fold activation over transfection without activator and expressed as the average of a typical triplicate experiment with error bars representing standard deviation. (**B**) HeLa cells were transiently transfected by pPSA-Luc and varying amounts of ERRα in presence (black bars) or absence (white bars) of XCT790. (**C**) LNCaP cells were treated with 5×10^{-6} M XCT790 for 48 h and 10^{-9} M DHT for 2 h, as indicated. The recruitment of ERRα to PSA gene regions was examined by ChIP analyses with anti-ERRα specific antibodies. IgG was used as control. Specific regions were amplified by QPCR. Results are expressed as percentage of input. Shown is the average of four independent experiments with error bars indicating standard deviation. ANOVA tests were performed to determine statistical significance; ns: nonsignificant, ***$P < 0.005$.

activated transcription from a construct (ARE-Luc) encompassing three androgen response elements (ARE) cloned in front of the minimal *collagenase* promoter (Figure 3B). DHT supplementation resulted in an additive effect, due to the endogenous expression of AR in HeLa

cells (as verified by western blot; data not shown). Importantly ERRα activated transcription even in the presence of the antiandrogen flutamide. As expected, AR activated transcription from the ARE-containing plasmid, an effect that was maximal upon DHT
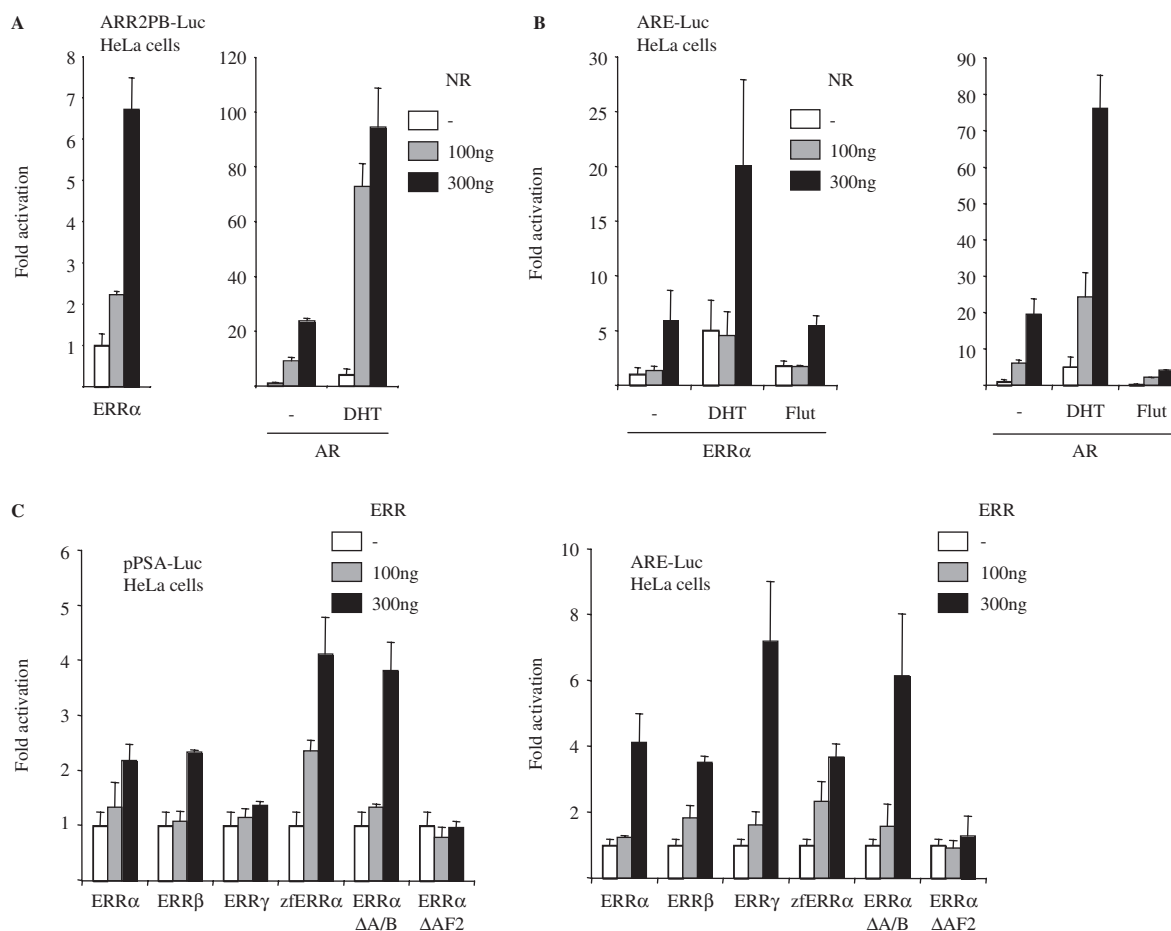
Figure 3. AREs confer responsiveness to ERRα. (**A**) HeLa cells were transfected with ARR2PB composite construct together with the indicated amount of ERRα- or AR-encoding plasmid. DHT (10^{-8} M) was added where indicated. (**B**) Same type of experiments using a construct in which three AREs were inserted in front of the minimal *collagenase* promoter. DHT (10^{-8} M) or flutamide (10^{-6} M) was added where indicated. (**C**) Cells were transfected with pPSA-Luc (left panel) or ARE-Luc (right panel) with the indicated ERR-encoding vectors. Except for zfERRα [zebra fish (*Danio rerio*) ERRα] all versions originate from mouse. All reporter activities were determined and expressed as in Figure 2A.

supplementation and blunted by flutamide addition. Altogether, this indicates that an ARE is sufficient to confer ERRα response to a heterologous promoter. Regulation through AREs was also extended to other members of the ERR subfamily (Figure 3C). Indeed, mouse ERRβ and γ, as well as zebra fish ERRα-activated transcription of the synthetic ARE-containing promoter. PSA promoter was also activated by mouse ERRβ and zebra fish, but apparently not by mouse ERRγ. Some nuclear receptors display two transcription activation functions, one (the AF1) located in the A/B (N-terminal) domain and the other (the AF2) located in the extreme C-terminal part of the molecule. On both reporter constructs, identical effects were observed using the wild-type or an A/B domain-deleted version of ERRα. In contrast, this receptor was inactive in the absence of the AF2 function. Thus, as for transactivation on other response elements (38), ERRα only requires the AF2 function to activate transcription from the ARE site.

In addition to its direct effect on ERRα, XCT790 could act on PSA expression by regulating the expression and/or activity of AR, a hypothesis that we next evaluated. The expression of AR-corresponding mRNA was

unaffected by treatment with XCT790, flutamide or a combination of both (Figure 4A). Likewise, AR protein level was not reduced upon drug treatment even after a 72 h exposure. In contrast, XCT790 dramatically reduced the ERRα protein level, but not of the corresponding mRNA, a phenomenon that we also have observed in several other cell lines (7). We next addressed whether XCT790 reduced the expression of androgen-responsive genes by merely blocking AR activity. ChIP experiments were first performed to determine whether AR recruitment to regions of the PSA promoter was modified by XCT790 treatment (Figure 4B). As expected, AR bound to the Enh-, as well as to the P- (although to a lower extent), but not to the E–P region of the PSA gene, in a DHT-dependent manner (49). Cotreatment by XCT790 had no effect on the recruitment of AR to the PSA promoter. We then reasoned that if XCT790 blocked AR activity on the PSA promoter, then DHT would be unable to up-regulate gene expression upon XCT790 pretreatment. On the contrary, DHT relieved the inhibition of PSA expression exerted by XCT790 (Figure 4C). Importantly DHT activates PSA expression to a similar extent in the presence or absence of XCT790 (round 2.5-fold). We thus concluded
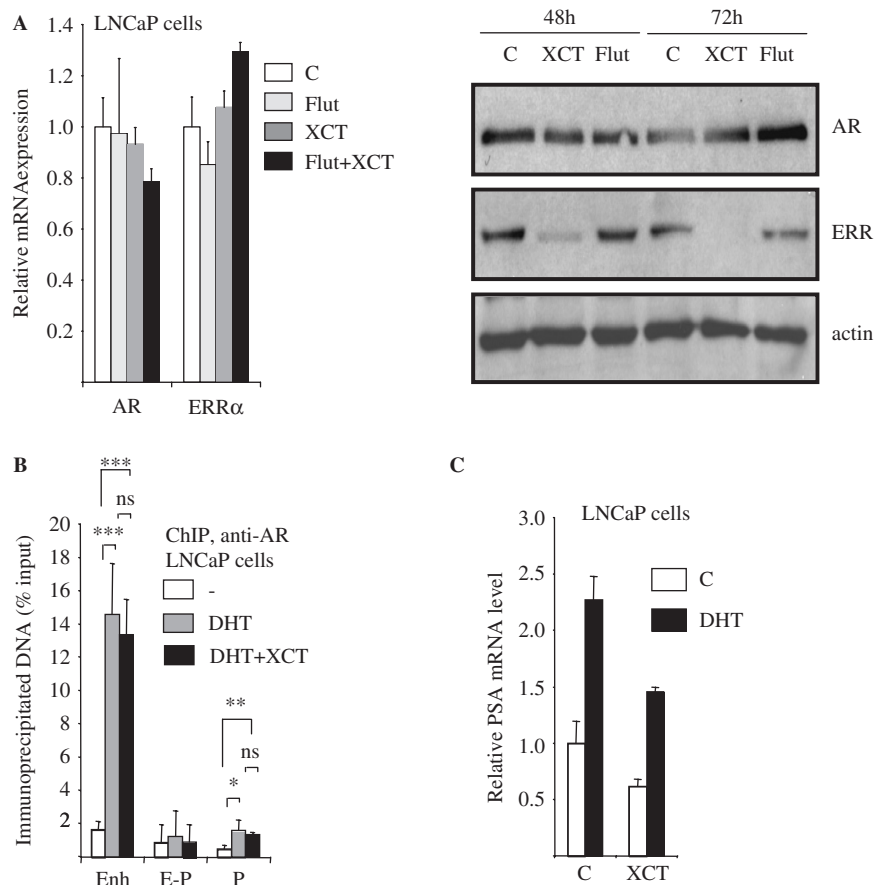
Figure 4. XCT790 does not impair androgen signaling. (**A**) LNCaP cells were treated with the indicated compounds. AR and ERRα mRNA expression were analyzed as in Figure 1A. Proteins were extracted and analyzed by western blotting using specific antibody for AR, ERRα or actin used as a loading control. (**B**) Cells were treated as indicated and ChIP were performed as in Figure 2B using anti-AR antibody. ANOVA test were performed to determine statistical significance; ns: nonsignificant, $^*P < 0.05$, $^{***}P < 0.005$. (**C**) Cells were treated with 5×10^{-6} M XCT790 (24 h pretreatment) and/or 10^{-8} M DHT (8 h treatment) as indicated. PSA mRNA expression was analyzed as in Figure 1A.

that XCT790 does not impact on AR expression or signaling.

ERRα activates transcription through AREs in HeLa and LNCaP cells, which both express AR. It is thus possible that AR is required for this effect of ERRα. To address this hypothesis, we performed cotransfection experiments in PC3 cells, which do not express AR (Figure 5A). In these cells, ERRα rather repressed the expression driven by the PSA promoter. In contrast, upon AR cotransfection, ERRα activated the PSA promoter in a dose-dependent manner. Strikingly, DHT was required for the transactivation by AR, but not for AR to complement ERRα activity. The same phenomenon was observed using the ARE-Luc construct. These results indicate that ERRα requires AR to activate transcription through ARE. In contrast, AR did not contribute to activation by ERRα of the pS2 promoter (which does not respond to AR), indicating a specific effect on androgen-responsive promoters. We next tried to determine the domains of AR involved in helping ERRα activation. Chimerae containing the AR-AF1 or -LBD fused to the Gal4 DBD did not complement ERRα activity on the PSA promoter indicating that these AR domains are not sufficient for complementation. We then compared the effect of GR derivatives. Cotransfection of wild-type GR

resulted in activation of the PSA promoter by ERRα. In contrast, GRdim which is defective in DNA binding (42), did not complement ERRα. This suggests that the DBD of StRs is necessary to complement ERRα-driven ARE transactivation.

In an effort to extend our findings to other steroid response elements, we next used the MMTV promoter, a well known model of steroid response. Upon cotransfection, ERRα by itself was inactive (Figure 6A). However, when supplemented with the PGC1-α coactivator, ERRα exerted a dose-dependent activation. The same profile was also observed using a construct in which GRE from the metallothionein promoter were cloned in front of the minimal *SV* promoter. In contrast, constructs harboring multimers of different steroid-unrelated response elements (LEF-RE, responding to TCF/LEF; DR1-Luc, responding to RAR-RXR; PerRE-Luc, responding to Period) were not activated by ERRα alone or in combination with PGC1-α (Figure 6A and data not shown), indicating that the effect is GRE specific. Activation is also ERRα specific, since both MMTV and GRE-Luc constructs were unable to respond to ERα, although the latter receptor was active on an estrogen response elements-containing plasmid (Figure 6A and data not shown). However, PGC1-α is not specific for
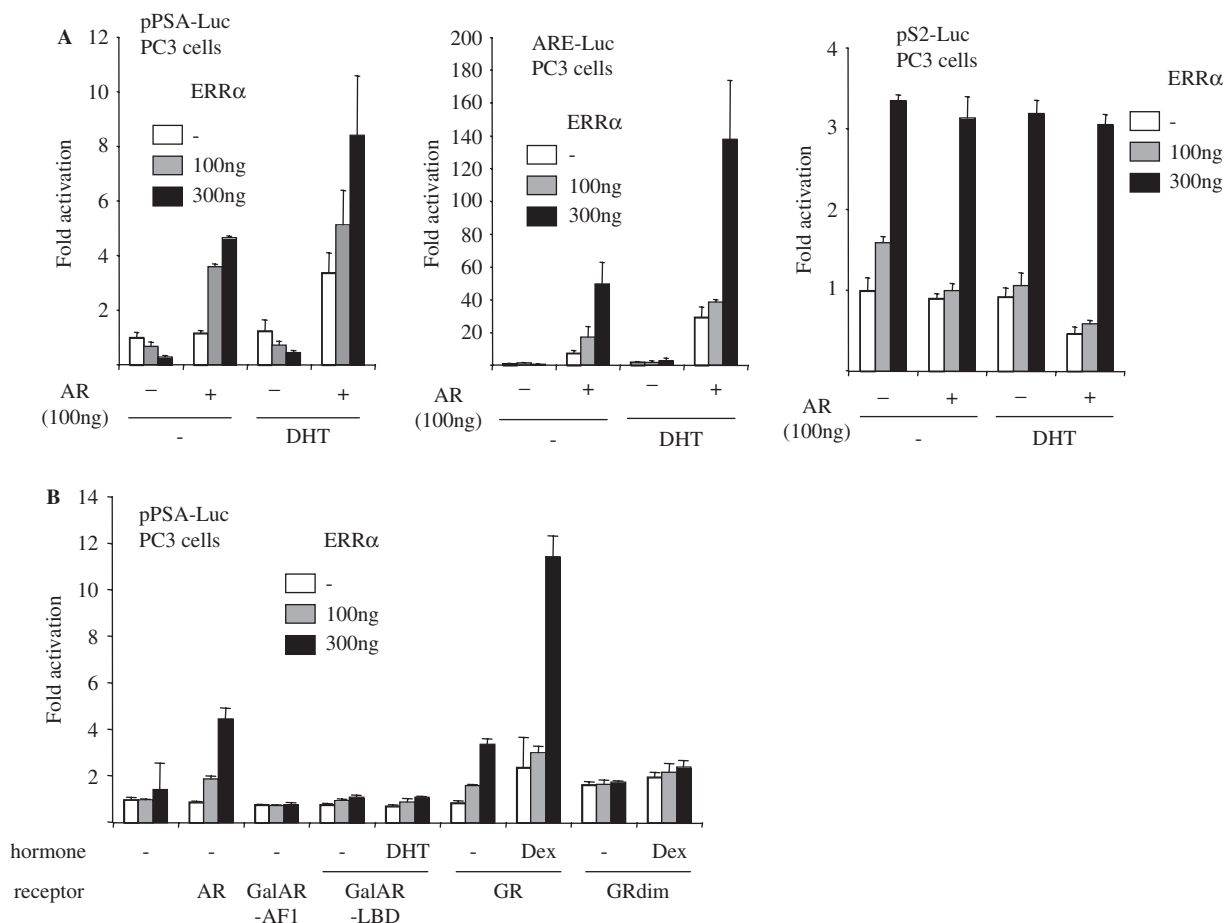
Figure 5. AR requirement in ERRα-induced ARE regulation. (**A**) PC3 cells were transfected with the indicated reporter plasmids and amounts of ERRα- or AR-encoding plasmids. DHT (10^{-8} M) was added where indicated. (**B**) Cells were transfected with pPSA-Luc together with the indicated amounts of ERRα and 100 ng of the indicated plasmids. All reporter activities were determined and expressed as in Figure 2A.

ERRα activity on the GRE, since cotransfection with SRC1 or GRIP1/TIF2 resulted in the same effect (Figure 5B).

Per definition, transcription from a GRE can be activated by the GR in the presence of glucocorticoids (such as the synthetic drug Dex), an effect that can be abrogated by the addition of synthetic antisteroid compounds such as RU486 (Figure 7A). In contrast, ERRα-PGC1-α activation through the GRE was unaffected by the presence of both Dex and RU486. This indicates that ERRα can activate transcription even in the presence of antisteroid compounds. In addition this also demonstrates that the effect of ERRα on the GRE does not result from activation of an endogenous StR, which would be blocked by RU486. In the experiments above, we observed that PGC1-α was capable of activating transcription through the GRE when transfected alone, and not only in the presence of exogenous ERRα. It is likely that, for this activity, PGC1-α requires a transcription factor as a DNA-tethering factor. To determine the nature of this factor, cells were transfected with GRE-Luc construct together with varying amounts of PGC1-α and subsequently treated with synthetic compounds (Figure 7B). The activity of PGC1-α was completely abrogated by

XCT790, but not by RU486 treatment, indicating that it depends on ERRα and not on an endogenous StR. However, an enhanced PGC1-α effect was observed when activating the GR pathway by Dex treatment. This over-activation could be specifically reversed when adding RU486. There again, XCT790 was required to completely abolish the transcriptional potency of PGC1-α. We thus concluded that PGC1-α is a promiscuous coactivator that can act through GR and ERRα. However in the absence of exogenously added hormones, PGC1-α activity on GRE is strictly dependent on ERRα.

DISCUSSION

Work by various laboratories including ours has pointed to several levels of interference between ERRα and estrogen signaling (27). For example, the recently determined *in vivo* ERRα-binding site (55) can also be used as an estrogen response element (28–32). In this report, we show that several genes that can be down-regulated by antiandrogens in LNCaP cells are also subject to repression by XCT790, an ERRα inverse agonist. This compound acts in a specific manner since it does not deactivate other ERRs, ERα nor AR (this report and
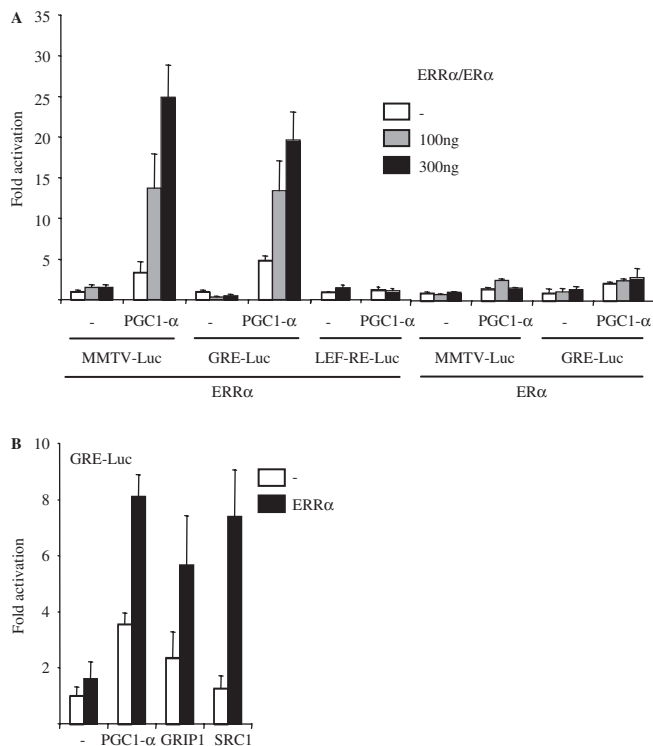
Figure 6. ERRs activate transcription through other steroid response elements in a coactivator-dependent manner. (**A**) HeLa cells were transfected with MMTV-Luc, GRE-Luc or LEF-RE-Luc constructs together with the indicated amount of ERRα or ERα. Where indicated, PGC1-α (100 ng) was added to the transfection mixture. In ERα transfections, 17β-estradiol (10^{-8} M) was added to the culture medium. (**B**) HeLa cells were transfected with GRE-Luc reporter plasmid together with the indicated coactivator, with or without ERRα.

Figure 7. Ligand modulation of ERRα/PGC1-α transcriptional effects. (**A**) HeLa cells were transfected with GRE-Luc together with GR, ERRα or PGC1-α, as indicated. The GR agonist Dex (10^{-8} M) or StR antagonist RU486 (10^{-6} M) were added to the culture medium as indicated. (**B**) HeLa cells were transfected with GRE-Luc reporter plasmid together with varying amounts of PGC1-α encoding plasmid. XCT790 (5×10^{-6} M), Dex (10^{-8} M) or RU486 (10^{-6} M) were added to the culture medium as indicated. All reporter activities in this figure were determined and expressed as in Figure 2A.

ref. 7). Furthermore, whereas the expression of *PSA*, *NKX3.1*, *KLK2* and *MAK* is down-regulated by XCT790, such genes as *AR*, *PGC1-α* or *ERRα* itself are not affected, indicating that the compound is not a general inhibitor of transcription. The effect of XCT790 is dependent on the presence of ERRα since the drug has no effect in the absence of the receptor.

Since XCT790 down-regulates the expression of various genes, we expected that overexpression of ERRα, brought about by adenoviral vector, would result in the opposite effect. However, in our hands, *PSA*, *NKX3.1* or *KLK2* did not respond to ERRα overexpression (data not shown). This can be due to a saturating effect of the endogenous ERRα protein on the promoters of these genes in LNCaP cells. Alternatively, a stimulating effect of ERRα could require the coexpression of a yet unidentified partner. Although active when transiently transfected alone, we also noted that the effect of ERRα on the PSA promoter is much lower than that of AR, suggesting that the orphan receptor is a weak activator, or that it lacks the coexpression of a potentializing partner for a maximal effect. The identity of this eventual partner is unknown but PGC1-α, bringing no further activation (data not shown), is not a likely candidate.

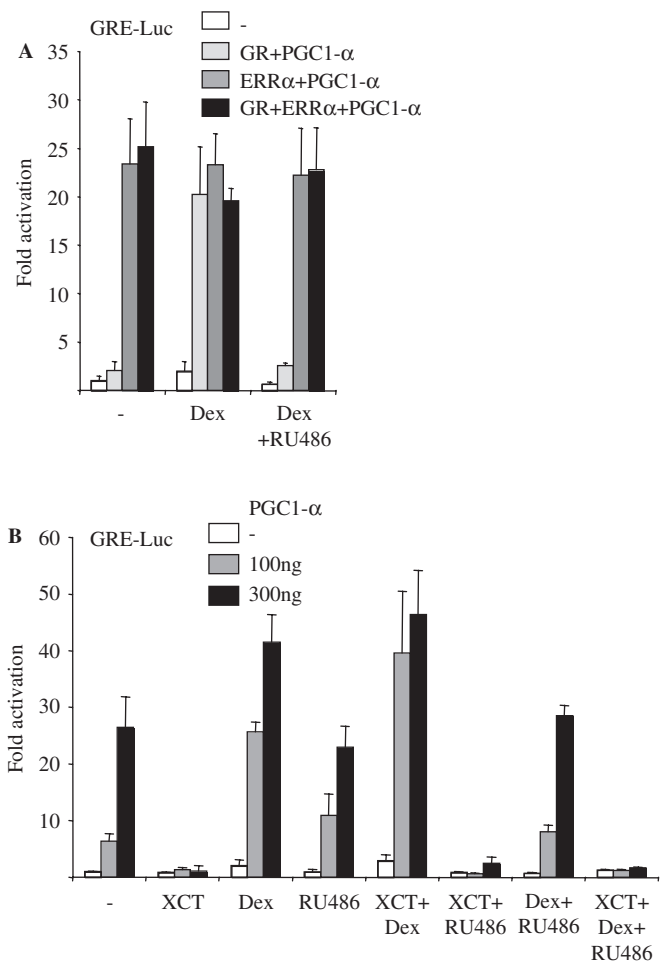In contrast to this, the effect of ERRα on other than-ARE StREs, requires the coexpression of a coactivator.

The effect of ERRα on StREs (ARE, GRE or MMTV) also extends to other members of the ERR subfamily, not only to all mouse ERR isoform, but also to zebra fish ERRα and ERRγ as well as to the unique ERR present in the amphioxus [(56,57); Figure 3C and data not shown]. It is thus likely that regulation through StREs is an ancestral property of all ERR receptors.

The regulation of expression exerted by ERRα through an ARE/StRE is at the promoter level, as evidenced by the fact that it can be transferred to a heterologous promoter in a specific manner. ERRα-responding ARE/StRE include derivatives from the PB, MMTV and metallothionein promoters (Figures 3 and 6) as well as from the nonpalindromic, AR-selective, GR-unresponsive human secretory component gene promoter (44) (data not shown). Given the variety of ARE/StRE used in this study, it seems likely that ERRα acts through this response element and not through cryptic ERREs that furthermore

could not be detected upon close inspection of the elements. However, direct binding of *in vitro* synthesized ERRα proteins on an ARE/StRE could not be observed in electrophoretic mobility shift experiments (data not shown), suggesting that the receptor may require an additional protein species to bind to ARE/StRE. The capacity to recognize these sequences depends on the P-box of the DBD (58–59). The P-box of ERRα is an ER-type (data not shown) which confers recognition of EREs but not of StREs. Consistently, we observed that ERRα was recruited to the PSA enhancer but to a lower extent than AR. XCT790 does not only deactivate ERRα but also rather transforms the receptor into an actively repressing factor. Two lines of evidence support this hypothesis. First, an siE does not down-regulate PSA expression, indicating that XCT790 does more than merely eliminating the receptor. Second, XCT790 promotes a higher level of ERRα recruitment to the PSA promoter. Although this may appear contradictory with XCT790-induced degradation of ERRα, it should be reminded that 17β-estradiol also promotes the degradation of its cognate receptor *and* its binding to DNA (60,61). Furthermore, binding of ERRα on the pS2 promoter in MCF7 cells is not diminished by XCT790 treatment (data not shown) although this also induces the degradation of the receptor in these cells (7). Thus the action of XCT790 at the local (promoter) level, as visualized by ChIP, appears different from its global (whole cell) effect observed by western blot.

Several factors have been identified that transcriptionally regulate androgen-responsive genes. In some cases, this regulation is exerted through perturbation of AR transcriptional activities. This is for instance the case of C/EBPα and Ebp1 that act as AR corepressors (62,63). In addition, Ebp1 also down-regulates the expression of AR itself providing an additional indirect control level on the expression of AR-dependent genes (47). None of these mechanisms are likely to explain the effects of the ERRα inverse agonist. Indeed, this compound does not perturbate AR expression or activity. This suggests that AR and ERRα act in parallel pathways. However, we did not observe any additive effect of antiandrogen and anti-ERRα cotreatment, which suggests that both drugs eventually act on a common factor. This apparent discrepancy can be solved when hypothesizing that ERRα requires AR to act on androgen-responsive promoter. In support to this, we observed that ERRα was capable of activating the PSA promoter in PC3 cells (which do not express AR) only upon AR supplementation. However, it should be noted that an active conformation of AR is not an absolute requirement. Indeed, ERRα transactivates through the ARE in the presence of the antiandrogen flutamide in PC3 as well as in HeLa cells (Figure 3B and data not shown). In PC3 cells, ERRα can also be complemented by GR to activate the PSA promoter. Interestingly, GRdim is inactive in ERRα complementation, indicating that DNA binding by a StR is involved. Consistently, (i) the AF1 or LBD moiety of AR are not sufficient to promote ERRα activity on the PSA promoter, (ii) ERRα does not promote the activity of GalAR-AF1 or GalAR-LBD on Gal4-binding sites (data not shown). Altogether, this suggests that ERRα does not directly bind to the ARE but

establishes transient contacts with a DNA-bound StR. XCT790 stabilizes these contacts and also induces a repressing conformation of ERRα, leading to promoter repression. Interestingly, this is unlikely to be mediated by HDAC recruitment.

Androgens play an essential role in prostate physiology. Prostate cancers are androgen-dependent for their growth, at least in the initial phases of the disease (64,65). Antiandrogen-based therapies are therefore widely used in the treatment of prostate tumors. Unfortunately, these tumors eventually develop resistance to antiandrogens, a phenomenon that lead to poor prognosis. Our results show that ERRα is able to stimulate the expression of androgen-responsive genes, even in the presence of anti-androgen. Our data may thus suggest that ERRα over-expression could help the cells to bypass the block exerted by antiandrogens. Through this, ERRα could play a role in the acquisition of androgen resistance in advanced prostate tumors. It has been shown that ERRα is indeed expressed in the prostate and in human prostatic cell lines (66). An eventual link between resistance to antiandrogen therapy and ERRα expression has not yet been investigated, but it should be noted that high ERRα expression is associated to poor prognosis in ovarian, breast and colon tumors (22–26). ERRα is active on ARE/StRE-containing promoters in the presence of the antisteroid synthetic compounds RU486 and flutamide. Our results lead to the hypothesis that a complete repression of such genes not only requires to inactivate the StRs but also the ERR family members.

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