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Estrogen receptor-α and Sp1 interact in the induction of the low density lipoprotein-receptor

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Abstract

Both estrogen and selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene have been demonstrated to lower plasma low density lipoprotein (LDL)-cholesterol concentrations by stimulation of LDL receptor gene expression. To determine the molecular mechanisms of estradiol- and tamoxifen-induced LDL receptor expression, we performed transient transfection experiments with luciferase reporter gene-constructs under transcriptional control of the human LDL receptor promoter. We demonstrate, that estradiol and tamoxifen stimulate LDL receptor gene expression in human HepG2 hepatoma cells only when estrogen receptor (ER)-α but not when ER-β is cotransfected. Deletion mutants and point mutations of the LDL receptor promoter reveal that estradiol- and tamoxifen-stimulated expression of this gene depends on an intact repeat 3 in the LDL receptor promoter, a *cis*-element previously shown to interact with Sp1. Gel mobility analyses demonstrated estradiol- and tamoxifen-stimulated binding of nuclear proteins to repeat 3 (bp −56 to bp −36) of the LDL receptor promoter. These data provide an alternative mechanism of LDL receptor gene expression by non-classical estradiol- and tamoxifen-stimulated induction through an ER-α/Sp1 complex.
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1. Introduction

Estrogen and selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene have been demonstrated to lower plasma low density lipoprotein (LDL)-cholesterol concentrations in vivo [1]. Further in vitro and in vivo studies have shown that estradiol stimulates expression of the LDL receptor gene [2]. Given the importance of circulating LDL-cholesterol concentrations as a major risk factor for coronary artery disease, the understanding of the molecular mechanisms of estradiol- and SERM-regulated LDL receptor expression will help to gain better insights into the regulation of cholesterol homeostasis.

The LDL receptor gene is a target of multiple signaling pathways. The best characterised mechanism of LDL receptor transcription is a sterol-sensitive pathway. Lowering of intracellular cholesterol concentrations activates sterol-sensitive proteases, which cleave the membrane-anchored transcription factors sterol-regulatory element binding protein (SREBP)-1 and -2 and allow the transcriptionally active N-terminal of these proteins to enter the nucleus. They activate transcription of the LDL receptor promoter through binding to and activating the sterol regulatory *cis*-element (SRE)-1 also termed repeat 2 in the regulatory region of the LDL receptor gene [3]. We have characterised an alternative mechanism, by which growth factors such as insulin, insulin like growth factor (IGF)-1 and platelet derived growth factor (PDGF) stimulate LDL receptor gene expression [4]. Activation of membrane bound tyrosine kinase receptors for these ligands leads to activation of the ras-raf-MAPkinase cascade resulting in the phosphorylation and subsequent activation of SREBPs without altering their nuclear abundance [5,6].

In contrast to these well characterised pathways the mechanism of estrogen-stimulated LDL receptor expression remained largely unclear. According to the classical model of estrogen action, after entering the cell,

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estradiol binds to its receptor in the cytoplasm of the cell. The ER is a member of the family of nuclear hormone receptors as a ligand-dependent transcription factor. Estradiol-binding leads to nuclear translocation, followed by estrogen receptor (ER)-dimerization and binding of estrogen-response-elements (ERE) in the promoter region of target genes [7]. A further consequence of ligand-induced ER-dimerization is the recruitment of transcriptional coactivators such as SRC-1 and transcriptional cointegrators such as p300/CBP resulting in transcriptional activation of the target gene [8,9]. More recently, an alternative ER has been identified and termed ER-β [10]. This receptor also binds estradiol (E2) and exerts similar effects at an ERE. Besides this well characterised mechanism of E2-regulated transcription, many target genes of estradiol lack a conserved ERE, and ERs have been demonstrated to act also through alternative *cis*-elements such as AP-1 and Sp1 sites [11]. More recently, Saville et al. have demonstrated an in vitro interaction of ER-α and -β with Sp1, leading to activation of a reporter plasmid under transcriptional control of a consensus Sp1-*cis*-element [12]. This finding is consistent with previous reports showing that a variety of physiologically relevant ER-target genes such as c-fos, cathepsin D, retinoic acid receptor 1a, adenosine desaminase, and insulin like growth factor binding protein 4 are regulated by ER-α/Sp1 interaction with the GC-rich promoter region of these genes [13,14]. Interestingly, while acting similarly through EREs, ER-α and -β regulate transcription through AP-1 and Sp1-sites differentially [11,12].

Also the promoter of the LDL receptor gene lacks a classical ERE-sequence, suggesting an alternative mechanism of ER-action on this gene. Here we demonstrate that both estradiol and tamoxifen stimulate LDL receptor gene expression selectively through activation of ER-α. We have characterised the estrogen-responsive element in the LDL receptor promoter in repeat 3, a cognate Sp1 binding site. Moreover, we show, that ER-α and Sp1 interact in vivo, and that both estradiol and tamoxifen stimulate recruitment of a Sp1-containing nuclear protein complex to repeat 3 of the LDL receptor promoter, defining a novel regulatory mechanism of LDL receptor gene expression.

2. Material and methods

2.1. Chemicals, biochemicals and plasmids

Charcoal treated fetal bovine serum (FBS) was obtained from Hyclone Inc. (Logan, UT). Fetal bovine serum, regular and phenolred-free RPMI 1640 medium, phosphate-buffered saline (PBS), estradiol, 4-OH-tamoxifen, 100× antibiotic/antimycotic solution were purchased from Sigma. γ-P^{32} ATP was obtained from Amersham. Expression plasmids for ER-α and -β have been previously described [15]. The estrogen-responsive reporter plasmid (ERE)2-TK was kindly provided by J. DiRenzo (Dana Farber Cancer Institute) and has been described previously. LDL-receptor reporter plasmids phLDL1, 4, 7 and the truncation mutants have been previously described [4]. The inactivation of repeat 1–3 either alone or in combination was performed by PCR after linearisation of plasmid phLDL4 with BbsI. Primers used for mutagenesis were as given further.

Repeat 1 : 5′-CGAAACTCCTCCTCTTGCAGTGAG GTGAAGACATTTGA-3′

Repeat 1mut : 5′-CGAAACTC*GAGA*TCTTGCAGTGA GGTGAAGACATTTGA-3′

Repeat 2/3 : 5′-AAATCACCCCACTGCAAA CTCCTCCCCCTGC-3′

Repeat 2/3mut : 5′-AAATCACCCCACTGCAAA CTCC*GAGAT*CTGC-3′

Repeat 2mut/3 : 5′-AAATCACC*G*CACTGCAAA CTCCTCCCCCTGC-3′

Repeat 2mut/3mut : 5′-AAATCACC*G*CACTGCAAA CTCC*GAGA*TCTGC-3′

After amplification the PCR reaction was digested with DpnI followed by religation. Plasmids were thereafter expanded and sequence-verified. Plasmid preparation kits have been supplied by Qiagen (Santa Clarita, CA). Forty percent polyacrylamide was obtained from National Diagnostics (Atlanta, GA)

2.2. Cell lines and transient transfection assays

HepG2-cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown and maintained in RPMI 1640 medium supplemented with 10% FBS. Prior to transfection, cells were cultured in phenolred-free RPMI 1640 medium supplemented with 10% charcoal-stripped FBS for 48 h. For transient transfections, cells were trypsinized, resuspended in electroporation buffer, electroporated with 0.29 kV, 500 μF and attached onto 6 well plates at a density of 3×10^5 cells per well. Six hour after transfection cells were stimulated with the indicated substances and left treated for 40 h. Thereafter, cells were collected in lysis buffer (Promega; Madison, WI) and the activities of firefly and ses panay (Renilla) luciferases were determined using the dual light system (Promega; Madison, WI). Assays were performed in triplicate and data presented are the mean ± S.E.M. of at least four individual experiments. Stimulation is expressed as fold-stimulation above unstimulated cells (set at 1).

2.3. Electrophoretic mobility shift assays (EMSA)

For electrophoretic mobility shift assays, 2×10^7 HepG2-cells were transfected with 20 μg of ER-α expression plasmid. After reseeding, cells were cultured in

phenol-red free RPMI-medium supplemented with 10% charcoal treated FBS. After stimulation with estradiol and tamoxifen for the indicated times, cells were scraped into PBS and collected by centrifugation. After resuspension in buffer A (20 mM Hepes/KOH pH 7.6, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.1% NP 40, 0.5 mM DTT, protease inhibitor complete (Boehringer; Mannheim, Germany), 5 mM KF, 1 mM glycerolphosphate, 1 mM Na vanadate) cells were lysed by repeated pipetting through a 27 G needle. Nuclei were pelleted by centrifugation and lysed in buffer B (20 mM Hepes/KOH pH 7.6, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$, 500 mM NaCl, 25% glycerine, 0.5 mM DTT, protease inhibitor complete (Boehringer; Mannheim, Germany), 5 mM KF, 1 mM glycerolphosphate, 1 mM Na vanadate). After repeated pipetting through a 27 G needle, nuclear extracts were subjected to centrifugation and stored at −80 °C. Oligonucleotides for gel shift experiments were LDL receptor repeat 3 (Sp1): 5′-TGCAAACTCCTCCCCCT-GCTA-3′, consensus-Sp-1: 5′- ATTCGATCGGGGCGGGGCGAGC-3′, mutant repeat 3: 5′-TGCAAACTCC*GAGAT*CTGCTA-3′. Annealed oligonucleotides (2 pmol) were 5′-end-labeled with 20 μCi γ-^{32}P ATP using 5 U polynucleotide kinase and purified using Sephadex G 50 colums. Three micrograms of nuclear extracts were diluted in 5× shift buffer (15 mM Hepes/KOH, pH 7.9, 10% glycerine, 1 mM EDTA, 1 mM DTT (final concentrations)) and 2 μg of dIdC/dAdT and bromphenolblue 0.01% were added to the reaction. 0.2 pmol (∼100,000 cpm) of the labeled fragment were added to the reaction and incubated for 20 min at RT. For competition experiments the indicated excess of the indicated oligonucleotides was added. Reactions were subjected to electrophoresis in 5% PAA-gels, vacuum dried and exposed to film. For supershift experiments, the indicated antibodies were incubated with the nuclear extracts for 1 h at 4 °C prior to addition of the radiolabeled probe.

2.4. Statistical analysis

Statistical analysis was performed using the SPSS- and Microsoft Excel software. For dose–response curve analysis an ANOVA test was performed for sigmoidal regression. Remaining statistical analysis was performed using an unpaired Student's *t*-test. The null-hypothtesis was rejected at a *P*-value <0.05.

3. Results

3.1. Estradiol and tamoxifen stimulate expression of the LDL receptor gene through ER-α

To analyse the mechanism of estradiol- and tamoxifen-stimulated LDL receptor gene expression, we performed gene reporter assays using a luciferase-reporter gene under transcriptional control of the regulatory region encompassing bp −537 to bp +88 of the human LDL receptor gene (Fig. 1A). When human HepG2 hepatoma cells were transfected in the absence of cotransfected expression plasmids for ER, both estradiol and tamoxifen failed to significantly induce transcription of this reporter gene, as previously described (data not shown), consistent with the finding that HepG2 cells in contrast to rat liver extracts do not express significant amounts of ER-α as determined by Western blot analysis (data not shown). Consistent with these findings Farsetti et al. could demonstrate that estradiol fails to activate transcription of an ERE-controlled reporter gene in the HepG2 cells in the absence of cotransfected ER-α.

When an expression plasmid for ER-α was cotransfected with the LDL receptor reporter gene, both estradiol and tamoxifen stimulated expression of the LDL receptor reporter construct in a dose-dependent manner with a maximally 3-fold stimulation (Fig. 1B). In contrast, when an expression plasmid for ER-β was cotransfected, both estradiol and tamoxifen failed to significantly induce LDL receptor promoter activity (Fig. 1B). These data indicate, that estradiol- and tamoxifen-induced activation of LDL receptor transcription is selectively mediated through ER-α.

3.2. Estradiol- and tamoxifen-inducibility of the LDL receptor gene is mediated through repeat 3 of its promoter

To identify the *cis*-element in LDL receptor promoter responsible for estradiol- and tamoxifen-stimulated transcriptional activation, we next employed truncation mutants of the LDL receptor promoter in transient transfection experiments (Fig. 2A). 5′-Deletions of the LDL receptor promoter up to bp −56, deleting both repeat 1 and 2 of the LDL receptor promoter had no effect on estradiol's and tamoxifen's ability to induce luciferase activity, indicating that activation occurs independent of the proximal Sp1-binding site (repeat 1) and the SRE-1 site (repeat 2) and elements further upstream up to bp −537 (Fig. 2A). Further deletion up to bp −36, deleting the 3′ Sp1-binding site (repeat 3), abolished estradiol- and greatly reduced tamoxifen-inducibilty of the reporter gene (Fig. 2A). To further confirm, that estradiol- and tamoxifen-inducibility of the LDL receptor promoter is mediated through the Sp1 binding site in repeat 3 of the promoter, we performed transient transfection experiments with point mutations in the promoter region. Therefore, repeat 1, repeat 2 and repeat 3—the characterised binding sites for factors acting in trans on the LDL receptor promoter—were mutated either alone or in combination. Consistent with the results obtained with the truncated reporter plasmids, isolated mutation of the SRE-1 site (repeat 2) in the construct phLDL 7 had no effect on estradiol- or tamoxifen-induction of transcriptional activation (Fig. 2B). Similarly, mutations of both repeat 1 and 2 in the construct hLDL 20 did not significantly alter induction of this promoter by either estradiol or tamoxifen (Fig. 2B). In contrast, isolated mutation of the Sp1 binding site in repeat 3 of the promoter in the construct phLDL 17 significantly reduced estradiol- and tamoxifen-induced stim-

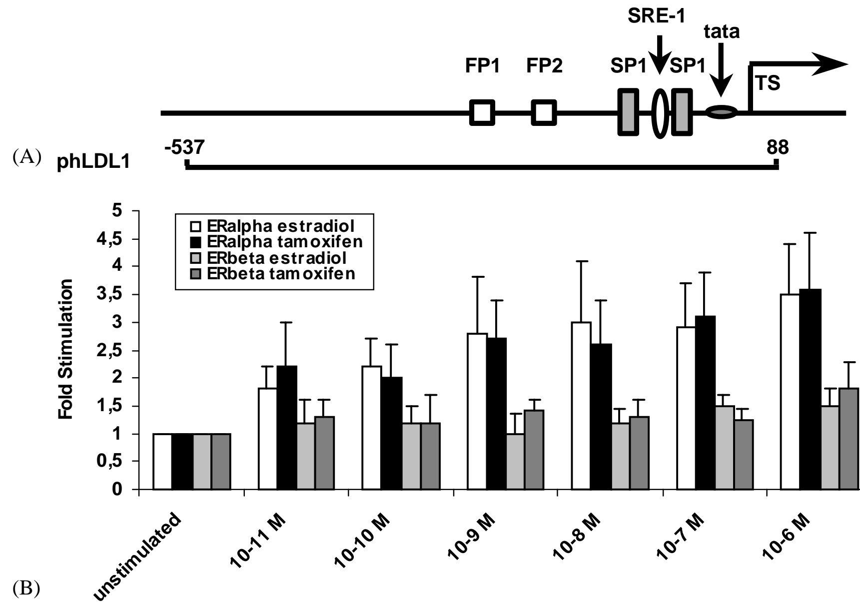


Fig. 1. Estradiol and 4-OH tamoxifen mediate activation of LDL receptor expression selectively through ER-α. (A) Schematic representation of the LDL receptor promoter luciferase reporter gene. (B) Comparison of ER-α- and ER-β-action from the human LDL receptor promoter. Six hours after transfection HepG2 cells were treated with either ethanol or estradiol or 4-OH tamoxifen at the indicated concentration and harvested 40 h later. Results are expressed as mean ± S.E.M. for four independent experiments with triplicate measurements and compared with controls (arbitrarily set at 1) cells treated with ethanol. Statistical analysis by ANOVA showed significance ($P < 0.05$) for sigmoidal regression of the dose–response curves, when ER-α was cotransfected. Moreover, unpaired Student's *t*-test revealed significant stimulation of transcription both through tamoxifen and estradiol for all doses used only when ER-α was cotransfected ($P < 0.05$). Within the different doses of estradiol stimulation of transcription was significantly different from 10^{-6} M vs. 10^{-11} and 10^{-10} M, from 10^{-7} M vs. 10^{-11} and 10^{-10} M and also from 10^{-8} M vs. 10^{-11} and 10^{-10} M (for all of these comparisons $P < 0.05$ in an unpaired Student's *t*-test). In case of tamoxifen-stimulation, transcriptional activity differed significantly within different concentrations from 10^{-6} M vs. 10^{-11} and 10^{-10} M ($P < 0.05$ in an unpaired Student's *t*-test).

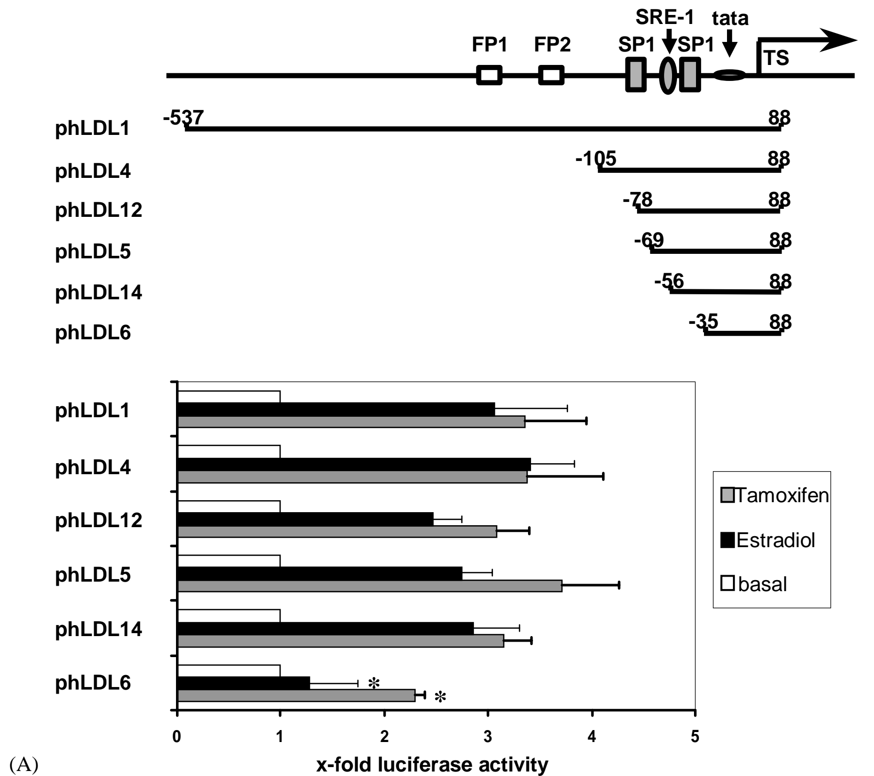
ulation of the LDL receptor promoter (Fig. 2B). Combined mutation of repeat 1 and 3 (phLDL 21) had no further effect on estradiol-stimulated transcription, but further reduced tamoxifen-stimulated LDL receptor expression as compared to the isolated mutation of repeat 3 in phLDL 17 (Fig. 2B). Taken together these experiments demonstrate, that both estradiol- and tamoxifen-inducibility of the LDL receptor promoter requires an intact Sp1-binding site in repeat 3 of the promoter. Although the isolated mutation of repeat 2 had no effect on estradiol- and tamoxifen-induced LDL receptor transcription, combined mutation of repeat 1, 2 and 3 in the construct phLDL23 completely abolished induction of the LDL receptor promoter by estradiol, and reduced transcriptional activation by tamoxifen, indicating that the SRE-1 site appears to co-operate with the distal Sp1-element (Fig. 2B).

3.3. Both transcriptional activation domains AF-1 and AF-2 of ER-α are required for estradiol- and tamoxifen-stimulated transcription of the LDL receptor gene

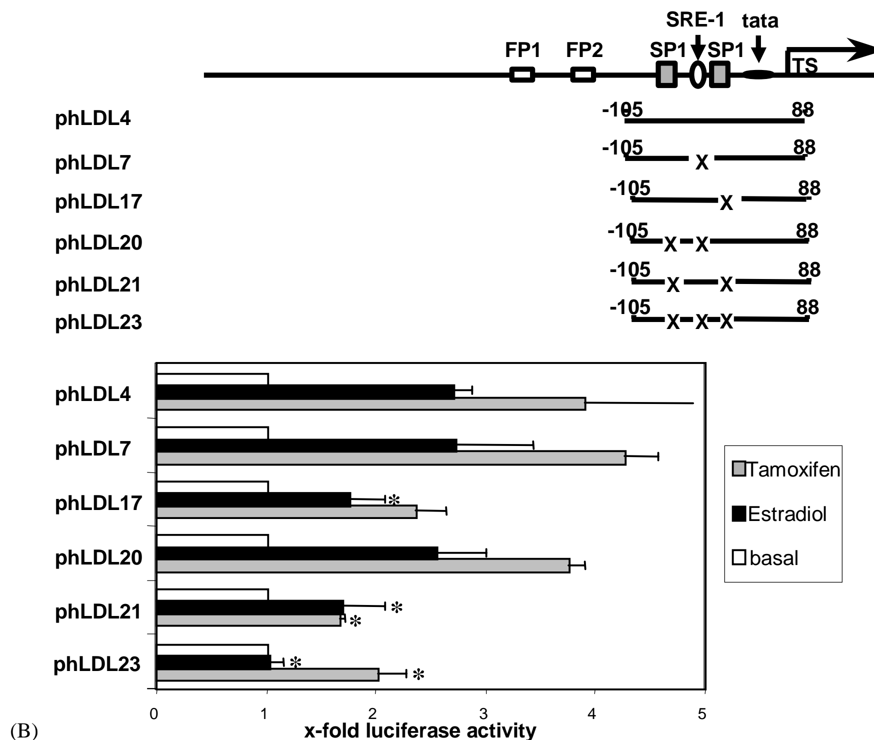
Since activation of the LDL receptor promoter is selectively activated through ER-α, we next investigated, which functional domains of ER-α are required for this effect. Similar to other members of the family of nuclear hormone

Fig. 2. Sp1-binding site in repeat 3 of the LDL receptor promoter mediates estradiol- and 4-OH tamoxifen-stimulated transactivation. (A) Identification of the estradiol- and 4-OH tamoxifen-response element in the LDL receptor promoter through 5′-deletion mutants of the promoter. The upper pannel shows a schematic representation of the truncated promoter constructs compared to the wild type promoter with the position of characterised *cis*-elements. Six hours after transfection with the indicated reporter constructs, HepG2-cells were treated with either ethanol or estradiol (10^{-8} M) or 4-OH tamoxifen (10^{-7} M) and harvested 40 h later. Results are expressed as mean ± S.E.M. for four independent experiments with triplicate measurements and compared with controls (arbitrarily set at 1) cells treated with ethanol. Student's *t*-test revealed significantly reduced transcriptional activion of phLDL6 both through estradiol and tamoxifen as compared to the remaining constructs ($P < 0.05$) (∗). (B) Identification of the estradiol- and 4-OH tamoxifen-response element in the LDL receptor promoter through point mutations of characterised *cis*-elements in the LDL receptor promoter. The upper panel shows a schematic representation of the mutant promoter constructs compared to the wild type promoter with the position of characterised *cis*-elements. Cells were treated and analysed as described in (A). Different results for construct phLDL4 compared to panel (A) result from the fact, that results were obtained in independent experiments. Transcriptional activity after estradiol-stimulation differed significantly from phLDL4 vs. phLDL17, phLDL21 and phLDL23 and from phLDL17 vs. phLDL23 ($P < 0.05$ in an unpaired Student's *t*-test) (∗). Moreover, transcriptional activity after tamoxifen-stimulation differed significantly from phLDL4 vs. phLDL21 and phLDL23 ($P < 0.05$ in an unpaired Student's *t*-test) (∗).

receptors, ER-α has two transcriptional activation domains, the N-terminal ligand-independent AF-1 domain and the C-terminal ligand-dependent AF-2 domain, which overlaps the hormone binding region [7]. To compare the role of these activation domains in classical ERE-mediated transcriptional activation to that of the LDL receptor gene, we performed transient transfection experiments of an ERE-controlled luciferase reporter gene, cotransfected with expression plasmids for wild type ER-α (ER-αWT) or two mutants lacking either the AF-1 domain or the AF-2 domain (Fig. 3A). As previously described in other cell lines, estradiol stimulated transcription of the ERE-reporter ∼3-fold,



(A)



(B)

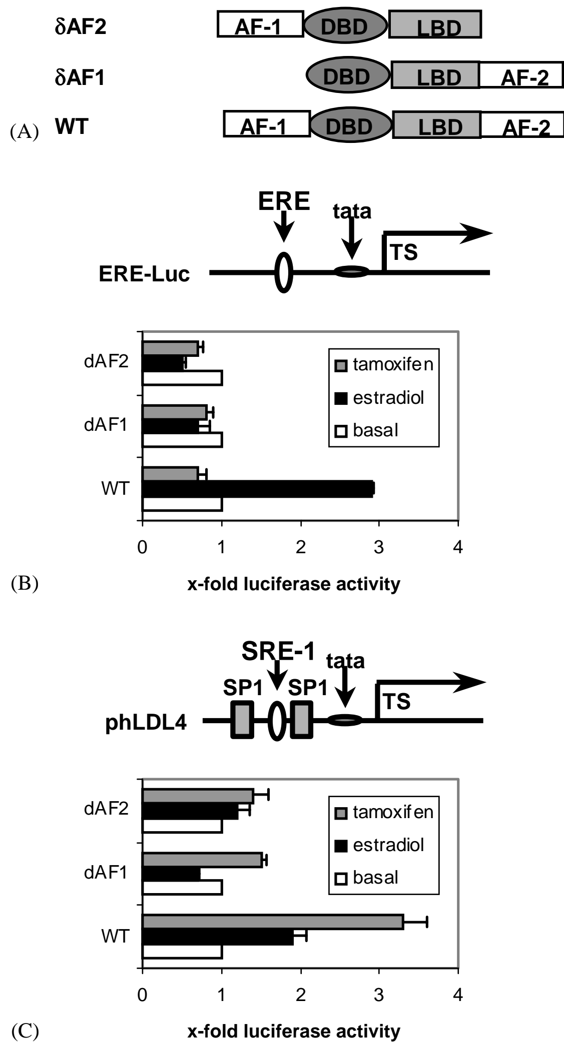


Fig. 3. Both transcriptional activation domains of ER-α are required for estradiol- and tamoxifen-stimulated LDL receptor transcription. (A) Schematic representation of wild type and mutant ER-α. Construct δAF-1 lacks aa. 1-123, δAF-2 lacks aa. 534-595 as compared to wild type ER-α (WT) (upper panel). (B) Comparison of ERE-mediated transactivation through wild type and mutant ER-α in HepG2-cells. Six hours after cotransfection of HepG2 cells with the ERE-controlled reporter plasmid and the indicated ER-expression plasmids cells were treated with either ethanol or estradiol (10^{-8} M) or 4-OH tamoxifen (10^{-7} M) and harvested 40 h later. Results are expressed as mean ± S.E.M. for four independent experiments with triplicate measurements and compared with control cells (arbitrarily set at 1) treated with ethanol. (C) Comparison of wild type and mutant ER-α-action on the LDL receptor promoter in HepG2-cells. Cells were treated and analysed as described in (B) with the exception that cotransfections were performed with the reporter plasmid phLDL4 instead of p(ERE)2-TK-Luc.

when an expression plasmid for ER-αWT was cotransfected (Fig. 3B). This transcriptional activation required both AF-1 and AF-2 activity of ER-α, since cotransfection of either mutant abolished estradiol-inducibility of the reporter plasmid (Fig. 3B). Tamoxifen acted as an ER-α antagonist on the ERE-controlled promoter as previously described in HepG2 cells, and mutations of either the AF-1 or AF-2 domain showed no further effect (Fig. 3B). Similarly, when ER-αWT was cotransfected with the LDL receptor promoter-controlled reporter gene, estradiol stimulated transcription ∼2.5-fold (Fig. 3C). This activation was abolished both by the mutation of the AF-1 domain and the AF-2 domain (Fig. 3C). Also tamoxifen-induced transcriptional activation of the LDL receptor promoter depended on the functional integrity of both transcriptional activation domains of ER-α (Fig. 3C). These data indicate, that in response to estradiol- and tamoxifen-stimulation both transcriptional activation domains of ER-α are required for LDL receptor gene expression.

3.4. Estradiol and tamoxifen stimulate binding of an ER-α/Sp1-containing protein complex to repeat 3 of the LDL receptor promoter

To further analyse, whether the interaction of ER-α and Sp1 has an effect on the binding of nuclear proteins to repeat 3 of the LDL receptor promoter, we performed electrophoretic mobility shift assays using radiolabeled repeat 3 of the LDL receptor promoter as a probe. Nuclear extracts were prepared from cells transiently transfected with the ER-α expression plasmid and left untreated or stimulated for 30 min, 1 and 4 h with estradiol. As shown in Fig. 4A, estradiol enhanced binding of nuclear proteins to the probe beginning after 30 min of stimulation, with maximal binding after 1 h of stimulation, and declining after 4 h of stimulation. Competition of binding with the unlabeled probe revealed specificity of DNA-binding (Fig. 4A). To test, whether this protein/DNA-interaction was mediated by the Sp1-binding site in repeat 3 of the LDL receptor promoter, we performed competition experiments with an unlabeled Sp1-consensus oligonucleotide. Also this oligonucleotide was able to compete for the estradiol-induced protein binding to the repeat 3 fragment, indicating that the Sp1 binding site indeed is responsible for recruitment of nuclear proteins to repeat 3 in response to estradiol-stimulation (Fig. 4A). These results were further confirmed, when excess of an unlabeled repeat 3 oligonuleotide with a mutation previously shown to abolish Sp1-binding was not able to compete estradiol-stimulated recruitment of nuclear proteins to the radiolabeled repeat 3 probe (Fig. 4A). Similar experiments were performed with nuclear extracts prepared from cells after tamoxifen-stimulation. Comparable to the results obtained for estradiol-stimulated cells, tamoxifen stimulated binding of nuclear proteins to a repeat 3 probe, and this binding was competed by either unlabeled repeat 3 fragment or a consensus Sp1-oligonucleotide, whereas the mutated oligonucleotide deficient in Sp1-binding failed to compete for specific binding (Fig. 4B).

To further analyse the nature of the protein complex recruited to repeat 3 upon estradiol-stimulation, we performed EMSA in the absence or presence of an anti-Sp1-specific antiserum. These experiments revealed a supershift of the estradiol-induced protein binding to repeat 3, whereas an

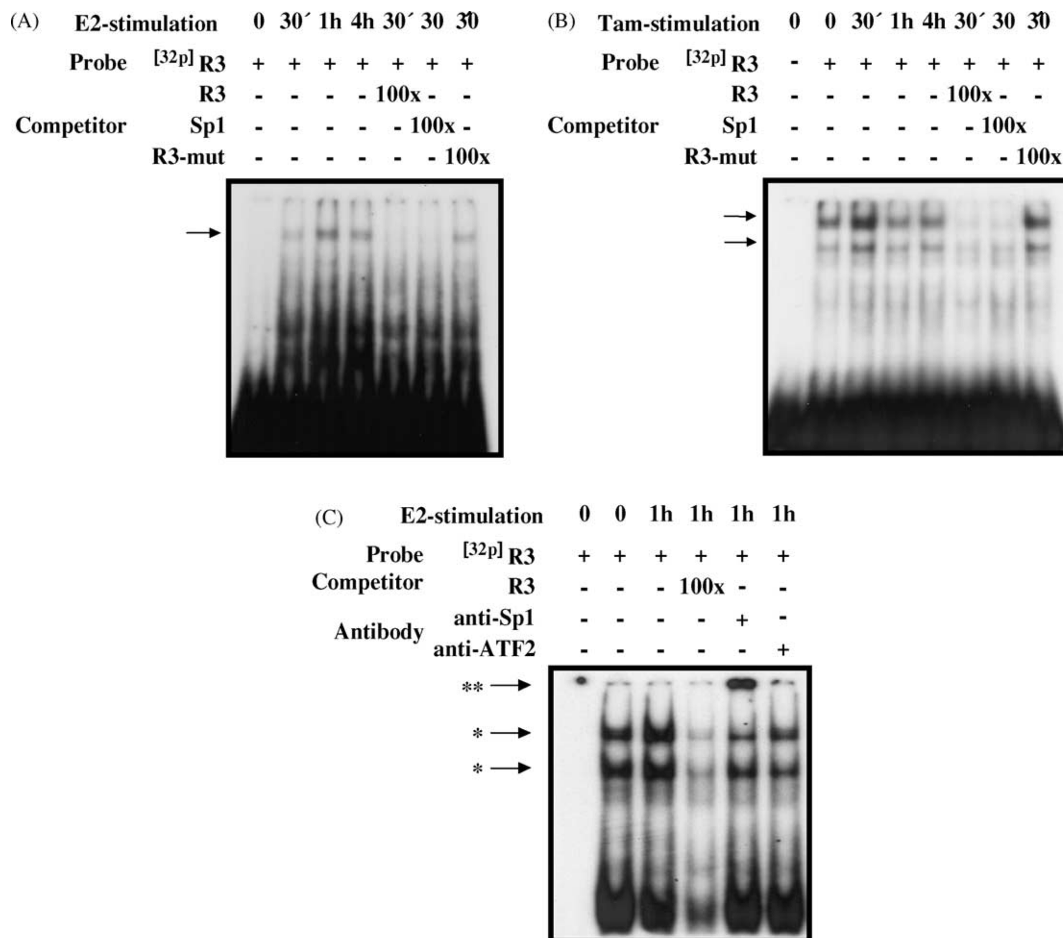


Fig. 4. Estradiol and tamoxifen enhance binding of nuclear proteins to repeat 3 of the LDL receptor promoter. (A) Nuclear extracts prepared from cells transiently transfected with an ER-α-expression plasmid were prepared after various times of estradiol-stimulation and incubated with a radiolabeled repeat R3 oligonucleotide (lanes 1–4). As a control, excess of either unlabeled R3 oligonucleotide (lane 5), unlabeled Sp1-consensus oligonucleotide (lane 6) or a mutant R3 oligonucleotide previously shown not to interact with Sp1 (lane 7) were added to the reaction. (B) Shows an experiment similar to that described in (A) with the exception that nuclear extracts were prepared from cells stimulated with 4-OH tamoxifen and that lane 1 represents a control of free probe in the absence of nuclear extracts. (C) Supershift analyses were performed using nuclear extracts as described in (B). Lane 1 shows a control of free probe in the absence of nuclear extracts, lanes 2 and 3 the incubation of radiolabeled R3-fragment with nuclear extracts from cells either without (lane 2) or after 1 h of estradiol-stimulation (lane 3). Lane 4 shows the specific competition by unlabeled R3-fragment. Lane 5 shows the supershift (**) of the bands induced by estradiol stimulation (*) by addition of a Sp1-antibody, while an ATF2-antibody fails to show a shift (lane 6).

unrelated antiserum to ATF-2 failed to cause a supershift of the repeat 3-bound protein, indicating that this protein indeed represents Sp1 (Fig. 4C).

4. Discussion

Cholesterol homeostasis is a major determinator for the integrity of the organism. While cholesterol is the precursor of steroid hormones it also is essential for maintenance of normal membrane function [16]. Therefore, intracellular cholesterol concentrations are tightly regulated through the interaction of multiple signaling pathways either regulating nuclear abundance of SREBP-1 and -2 or targeting their transactivation activity through growth factor-stimulated phosphorylation [3–6]. While both of these signaling pathways leading to LDL receptor expression are mediated through SREBPs and the SRE-1 site in the LDL receptor promoter, we describe a mechanism of estradiol- and tamoxifen-stimulated LDL receptor expression mainly mediated through a Sp1-binding site in repeat 3 of the LDL receptor promoter.

Transcriptional activation of genes by nuclear hormone receptors such as ER is complex and mediated through recruitment of multiple proteins to the promoter of target genes by the activated hormone receptor. Moreover, additional complexity of estradiol and tamoxifen signaling is achieved by the existence of functionally overlapping but also distinct receptor isoforms and isotypes [10,15]. While acting on ERE-regulated target genes, the liganded ER binds directly to DNA, on alternative *cis*-elements the ER modulates transcription through protein/protein interaction

with other transcription factors without getting into direct contact to DNA [12]. Indeed many estrogen-regulated genes lack classical ERE-sequences and estradiol regulation of these genes is mediated through alternative *cis*-elements such as AP-1 and Sp1 sites. This mechanism adds additional complexity to ER-mediated transcriptional regulation, since various ligands of ER can exert distinct effects by acting through different *cis*-elements [11]. It has been shown, that in HeLa cells both estradiol and tamoxifen stimulate ER-α-mediated transcription through AP-1 sites, whereas only tamoxifen stimulates AP-1-activity through ER-β in the same cell line. Similarly, Saville et al. could demonstrate that estradiol and tamoxifen stimulate ER-α/Sp1 in MCF-7, LnCaP, and MDA-MB-231 cells, whereas ER-β/Sp1 fails to do so in the same cells [12]. Moreover, a very recent report by Li et al. describes the activation of the LDL receptor promoter through ER-α/Sp1 complexes [17]. Although this group did not investigate the role of ER-β and tamoxifen-induction on LDL receptor transcription, their findings are consistent with ours, that ER-α can activate transcription of the LDL receptor promoter through Sp1-interaction. We demonstrate, that this effect is specific for ER-α, since ER-β is not capable of mediating this effect. Some insights into the mechanism of differential Sp1-activation by ER-α and -β have been added from the use of chimeric ER-α/-β receptors. While the ER-α AF-1 domain refers Sp1-inducibility to ER-β on a consensus Sp1-reporter gene, the AF-1 domain of ER-β inactivates ER-α on Sp1-elements, while physical interaction of Sp1 was detectable with both ER-α and ER-β in vitro [12]. In contrast to Li et al., our results indicate, that both the AF-1 and the AF-2 domain of ER-α are required for ER-α/Sp1-mediated activation of the LDL receptor gene. In the context of the action of chimeric receptors on a Sp1-element as described by Saville et al., we conclude, that binding of Sp1 occurs in the AF-2 region of ER-α and -β, whereas cofactors specific for the ER-α AF-1 domain mediate transcriptional activation of the ER-α/Sp1 complex. It is also interesting that known AF-2-coactivators such as SRC-1 failed to enhance ER-α/Sp1 activation (data not shown), indicating that indeed the AF-2 domain does not exert its classical transcriptional activation as known from ERE-elements. Therefore, ER-α-mediated transcriptional regulation through GC-rich regions occurs through at least two mechanisms, one is the receptor mediated enhancement of Sp1-binding to the *cis*-element, another being the recruitment of specific coactivators to this complex. Therefore, the ER-α/Sp1-mediated activation of LDL receptor transcription supports the model proposed by Saville et al. for ER-α/Sp1 action on a consensus Sp1-promoter. While we could detect coimmunoprecipitation of ER-α and Sp1 in vivo (data not shown) pointing to the possibility, that this protein/protein interaction enhaces Sp1/DNA-binding, we cannot rule out that estradiol-stimulated posttranslational modifications of Sp1, such as phosphorylation induce the enhancement in Sp1/DNA-binding.

The role of SREBPs and the SRE-1 *cis*-element in estradiol and tamoxifen-stimulated LDL receptor transcription remains controversial. While both, the isolated point-mutation of the SRE-1-site and the results obtained from the truncation mutants do not support a role for the SRE-1-site in estrogen- and tamoxifen-regulated activation of LDL receptor transcription, in the context of an intact repeat 3 element, combined mutations of the SRE-1-site and Sp1-elements result in a further reduction of transcriptional activation as compared to the isolated repeat 3-mutation. Similarly, in the context of an intact repeat 3 in the LDL receptor promoter, we could previously demonstrate, that expression of SREBP-antisense mRNAs has no effect on estradiol-induced LDL receptor transcription [4]. These data could direct to a role of SREBPs and the SRE-1-site in the context of impaired ER-α/Sp1 interaction or inactivation of repeat 3, but could also point to a role for Sp1/SREBP interaction required for full transcriptional activation as it has been previously demonstrated [18].

While previous work by other groups had demonstrated the physiological relevance of ER-α/Sp1- regulation for genes involved in the regulation of tumor growth such as c-fos, cathepsin-D, retinoic receptor 1a, E2F1, bcl and IGF-BP4, our findings extend the importance of this mechanism to the regulation of a key player in metabolic homeostasis.

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