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Sp1 transcription factor expression is regulated by estrogen-related receptor α1

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Abstract

The current study examines the relationship between ERRα1 and the Sp1 transcription factor. The transfection of ERRα1 enhances Sp1–DNA complex formation as visualized by electrophoresis mobility shift assay (EMSA). In addition, luciferase activity under the control of three consensus sites for Sp1 binding is activated by ERRα1 transfection. By looking further upstream from this point we have found that ERRα1 stimulates the upregulation of Sp1 protein expression by activating its transcription. Indeed, human Sp1 promoter-dependent luciferase activity is activated by ERRα1 transfection and a DNA–protein complex is observed by EMSA using oligonucleotides encompassing the putative ERRα1 binding site on the human Sp1 promoter (−1444/−1433: 5′-AGGACATGACCT-3′). In addition, activation of Sp1 promoter-dependent luciferase activity depends on the A/B and C domains of ERRα1 as shown with truncated ERRα1 cDNAs. This report is the first to demonstrate that ERRα1 promotes Sp1 activity through upregulation of Sp1 expression at the transcriptional level.

Keywords: Estrogen receptor; Estrogen-related receptor; Sp1; Transcriptional factor; Gene expression; Transcription

Estrogen-related receptor α (ERRα) was discovered by a low-stringency cloning technique using the DNA binding domain of estrogen receptor α (ERα) [1]. Subsequent investigation showed that ERRα can be translated from the second Met in the ERRα gene to produce the fully active ERRα1 [2,3]. Although ERRα is an orphan receptor whose ligands are unknown, some recent investigations show that ERRα interacts with members of the peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) family leading to ERRα transactivation activity and also a regulation of ERRα expression [4,5]. In addition, ERRα has been implicated in the control of energy balance and in the progression of diabetes, as ERRα expression seems to be closely related with PGC-1 and medium chain acyl-CoA dehydrogenase (MCAD), which catalyze the initial step in mitochondrial fatty acid oxidation [5–7]. Although PGC-1 seems to activate ERRα transactivation like a ligand, the existence of small hydrophobic natural ligands for ERRα is highly probable. Indeed, flavone (6,3′,4′-tri-hydroxyflavone) and isoflavone (biochanin A, genistein, and daidzein) phytoestrogens activate ERRα transactivation [8], and ERRα transactivation activity is not detected in cells cultivated in charcoal-stripped serum [9]. We have demonstrated that estrogen response element (ERE)-dependent luciferase activity is not enhanced by ERRα1 transfection in bovine pulmonary endothelial cells (BPAECs) that have been treated with charcoal-stripped serum [10].

ERRα1 belongs to the nuclear receptor superfamily which shares a highly conserved domain organization. In the nuclear receptor, generally, the A/B domain is located in the N-terminal and is necessary for the hormone-independent transactivation function (AF-1) while the C domain has a binding capacity for specific regions of DNA. The D domain forms a bridge between the C and E domains as well as providing a signal for

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nuclear localization. Finally, the E domain is a ligand-binding domain and includes the hormone-dependent transactivation function (AF-2).

Endothelial nitric oxide synthase (eNOS) expression and activity play an important role in vascular homeostasis and the inhibition of atherosclerosis development [11]. It has been shown that the estrogen–ERα pathway upregulates eNOS expression [12], stabilizes eNOS mRNA [13], and stimulates eNOS phosphorylation, leading to activation [14]. In a previous study, we have shown that ERRα1 transfection upregulates endogenous eNOS expression and enhances eNOS promoter-dependent luciferase activity in BPAECs. Although we proposed that two steroidogenic factor 1 response elements (SFRE) are necessary for the ERRα1 transactivation of the eNOS promoter (−805: 5′-TCAAGCTCT-3′: −796 and −593: 5′-TCAACCACA-3′: −585), we did not detect a decrease in ERRα1 transactivation of the eNOS promoter by several mutations in the SFREs [10].

It has been shown that estrogen stimulates eNOS expression through the ERα-ERE and Sp1 in endothelial cells [15] and EA.hy926 cells [16]. There have been two mechanisms proposed to explain how estrogen activates Sp1 and thus upregulates eNOS expression. One possibility is that an Sp1–ERα protein interaction may upregulate eNOS expression, as such a protein interaction has been shown to regulate heat shock protein 27, c-*fos* or low-density lipoprotein receptor expression [17–19]. The other proposed mechanism is that Sp1 expression is upregulated by estrogen, as a predicted ERE exists in the reported human Sp1 promoter [20]. However, these possibilities still remain to be investigated and, in addition, the relationship between ERRα1 and Sp1 is also unknown.

In this report, we show that ERRα1 stimulates the formation of an Sp1–DNA complex as well as the induction of Sp1dependent-luciferase activity. In addition, Sp1 expression is upregulated at the transcriptional level by ERRα1 transfection.

Materials and methods

Plasmid construction. The 3 times Sp1–Luc and 3 times mutated Sp1–Luc consisting of three consensus or mutant Sp1 binding sites were generated with the following DNA oligos, 5′-CGCGTGGGCGGAACTGGGCGGAGTTAGGGGCGGGA-3′ and 5′-AGCTTCCCGCCCCTAACTCCGCCCAGTTCCGCCCACGCG-3′ or 5′-CGCGTG*TTTTT*GAACTG*TTTTT*GAGTTAGG*TTTTT*GGA-3′ and 5′-AGCTTCC*AAAAA*CCTAACTC*AAAAA*CAGTTC*AAAAA*CACGCG-3′ (italic letters indicate the bases for mutation). The annealed oligos were inserted into pGL2-Basic (Promega, WI) which had been cleaved with *Sma*I and *Hin*dIII restriction enzymes. The human ERRα1 (1–423) was generated as previously described [10]. To make two truncated cDNAs: ERRα1 (79–423) and ERRα1 (144–423), we performed PCR with Platinum High Fidelity *Taq* DNA Polymerase (Invitrogen, CA) using, the sense primers (79–423): 5′-TAAAT<u>ATG</u>**GCA**TGCCTGGTCTGTGGGGACGTGGCCTCC-3′ and (144–423): 5′-GTAAT<u>ATG</u>**GCA**ATGCTCAAGGAGGGAGTGCGCCTGGAC-3′ (underlined letters indicate the translation initiation codon, and bold letters indicate bases which optimize translation to produce the truncated ERRα1 protein controlled by pcDNA3.1(+)). The antisense primer: 5′-GCCGAATTCCTTGCCTCAGTCCATCATGGC-3′ was used for making both plasmids. ERRα1 (1–423) cDNA was used for template DNA. Amplified cDNAs were cloned into pCR-TOPO4 (Invitrogen, CA). Truncated ERRα1 cDNAs were sequenced and subcloned into pcDNA3.1(+) to construct ERRα1 (79–423)/pcDNA3.1(+) and ERRα1 (144–423)/pcDNA3.1(+) for high expression of truncated ERRα1 in transfected cells. Two Sp1 promoter-fused luciferase cDNAs (pGL3-FOR5 and pGL3-FOR4) were kind gifts from Dr. Carlos Ciudad (University of Barcelona). ERE-luciferase cDNA was a kind gift from Dr. Donald McDonnell. ERα/pcDNA3 cDNA was a kind gift from Dr. Hermes Garban.

Cell culture. COS-7 cells were obtained from the ATCC (Manassas, VA). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Transient transfection and luciferase assay. To investigate whether ERRα1 activates binding against consensus estrogen response element (ERE), Sp1 and AP-1, and also to establish whether ERRα1 upregulates Sp1 protein expression, 4×10^5 COS-7 cells were seeded in 6-well plates. Transfection was performed with Polyfect (Qiagen, CA) according to the manufacturer's protocol. COS-7 cells were transfected with 1.5 μg of indicated cDNA for 48 h.

To investigate ERRα1 transactivation activity, 2×10^5 COS-7 cells were seeded in 12-well plates. Transfection was performed as previously described. COS-7 cells were transfected with 1 μg reporter cDNA, 0.1 μg receptor cDNA, and 0.1 μg β-galactosidase cDNA. Transfected cells were washed with PBS and lysed with luciferase assay system lysis buffer. Luciferase activity was measured in cellular extracts using the luciferase assay system (Promega, WI). To correct for transfection efficiency, luciferase activity was normalized to the corresponding β-galactosidase activity, which was measured by the β-galactosidase assay system (Promega, WI).

Production of anti-ERRα1 polyclonal antibody. Antibodies were raised in rabbits with designed and HPLC purified peptides (residues: 363–377, EAGRAGPGGGAERRR) covalently coupled to a carrier protein, KLH (Synpep, CA). The immunogen was injected subcutaneously with Freund's complete adjuvant. The antibody titer in antiserum was monitored by ELISA.

Electrophoresis mobility shift assay (EMSA). The nuclear extracts of transfected cells were extracted with the NE-PER nuclear extraction reagent (Pierce, IL). Double stranded oligonucleotides encompassing the ERE (Santa Cruz Biotechnology, CA), Sp1 and AP-1 (Promega, WI) were end-labeled using [γ-^{32}P]ATP, 7000 Ci/mmol (MP Biomedicals, CA) and T4 polynucleotide kinase (Promega, WI). Also, annealed oligonucleotides for the human Sp1 promoter region from −1452 to −1427 (5′-AATACTGA**AGGACATGACCT**CATCGT-3′) were used as a DNA probe (bold letters indicate the putative ERRα1 binding site). Five microgram of nuclear protein extract was incubated for 30 min at room temperature in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, and 50 μg/ml poly(dI–dC)), 100,000 cpm radiolabeled consensus oligonucleotide was subsequently added to each sample, and incubated at room temperature for an additional 30 min. Protein–DNA complexes were subsequently resolved in a 7% native TBE (Tris–borate–EDTA) gel. Gels were dried and exposed to autoradiographic film at −80 °C.

Western blot analysis. Twenty micrograms of protein was separated by 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Gels were transferred to a nitrocellulose membrane and then placed in blocking solution (TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) and 5% nonfat milk) for 1 h. Blots were incubated for 1 h with anti-ERRα1 polyclonal antibody, anti-Sp1 polyclonal antibody, anti-ERα monoclonal antibody (Upstate Biotechnology, NY) or anti-actin monoclonal antibody (Chemicon, CA), washed with TBST, and incubated with HRP-conjugated secondary

antibody. Bound IgG was visualized using an enhanced chemiluminescence detection system (Pierce, IL) according to the manufacturer's protocol. Band intensities were quantified with NIH-image software.

Statistical analysis. Data were obtained from three or four separate experiments. Each value represents the mean ± SEM. Statistical significance was assessed by Student's t test for unpaired values, and differences between treatment groups were considered statistically significant at $p < 0.05$.

Results

ERRα1 stimulates binding against Sp1 consensus site

In our previous report, we showed that ERRα1 transfection upregulates eNOS expression at the transcriptional level [10]. In order to investigate which transcription factors regulate ERRα1-enhanced eNOS expression, we performed EMSA with consensus Sp1 and AP-1 oligonucleotides, transcription factors which are known to regulate eNOS expression. To confirm the experimental system, we began by performing EMSA with a consensus ERE oligonucleotide and nuclear extract obtained from vector or ERRα1 (1–423)/pcDNA3.1(+) cDNA-transfected COS-7 cells. As shown in Fig. 1, ERRα1 transfection resulted in a shift of the ERE oligonucleotide as compared with vector transfection alone. We observed that ERRα1 transfection enhances Sp1–DNA complex formation compared with vector transfection, however, the binding of the AP-1 oligonucleotide was not enhanced by ERRα1 transfection. The specificity of binding was confirmed in the presence of excess unradiolabeled oligonucleotide for each of the probes (Fig. 1, lane 3).

ERRα1 activates Sp1-dependent luciferase activity

To understand the relationship between ERRα1 and the transcription factor, Sp1, we examined whether the expression of ERRα1 activates consensus Sp1-dependent luciferase. We co-transfected ERRα1 (1–423)/pcDNA3.1(+) and a promoter/reporter construct consisting of three consensus binding sites for the Sp1 transcription factor fused to luciferase (3 × Sp1–Luc) into COS-7 cells. Fig. 2 shows that ERRα1 transfection significantly enhanced Sp1-dependent luciferase activity (white bar). This effect was lost when the binding sites for Sp1 were mutated (3 × mutated Sp1–Luc; black bar). A similar effect was observed in transfected HELAcells (data not shown).

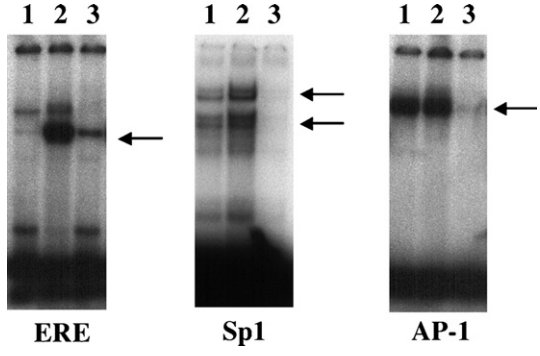

Fig. 1. EMSA for ERE, Sp1, and AP-1. Five micrograms of nuclear extracts that were obtained from pcDNA3.1(+) (lane 1) or ERRα1 (1–423)/pcDNA3.1(+) (lane 2)-transfected COS-7 cells was reacted with consensus ERE, Sp1 or AP-1 oligonucleotides. DNA–protein complexes were confirmed in the presence of 100-fold excess unradiolabeled oligonucleotides (lane 3).

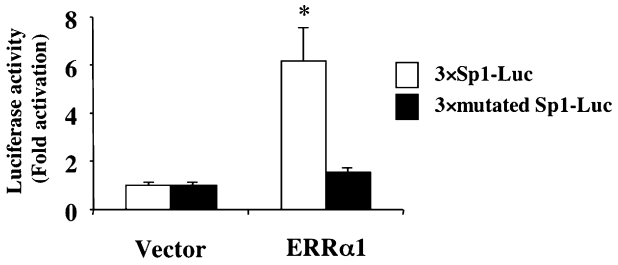

Fig. 2. Sp1 consensus binding site-dependent luciferase activity driven by ERRα1 in COS-7 cells. ERRα1 (1–423)/pcDNA3.1(+) or pcDNA3.1(+) were transiently co-transfected with 3 × Sp1–Luc (white bar) or 3 × mutated Sp1–Luc (black bar) cDNA into COS-7 cells. Cells were cultured for 48 h. Sp1-dependent luciferase activities were determined and were plotted as fold activation over the individual luciferase activity with vector (*$p < 0.05$ vs vector).

ERRα1 upregulates endogenous Sp1 protein expression

To investigate how ERRα1 enhances the formation of an Sp1–DNA complex and activates Sp1-dependent luciferase activity (Fig. 2), we studied endogenous Sp1 protein expression in ERRα1-transfected COS-7 cells by Western blot. As shown in Fig. 3, endogenous Sp1 protein expression was upregulated at both 24 and 48 h after ERRα1 transfection. The most significant effect on Sp1 was observed at 48 h which corresponded with the peak expression of transfected ERRα1.

ERRα1 activates Sp1 promoter-dependent luciferase activity

To examine whether ERRα1 upregulates Sp1 expression at the transcriptional level, we co-transfected ERRα1 (1–423)/pcDNA3.1(+) and human Sp1 promoter-fused luciferase (pGL3-FOR5 or pGL3-FOR4, Fig. 4A). As shown in Fig. 4B, the pGL3-FOR5 (human Sp1 promoter (−1612/−20)-fused luciferase)-dependent luciferase activity was enhanced by ERRα1 transfection (2.08-fold) and pGL3-FOR4 (human Sp1 promoter (−751/−20)-fused luciferase)-dependent luciferase activity was also enhanced (1.63-fold). There was a significant difference between the enhancement of pGL3-FOR5 and pGL3-FOR4-dependent luciferase activity by ERRα1 transfection, with a greater effect observed h the longer promoter sequence.

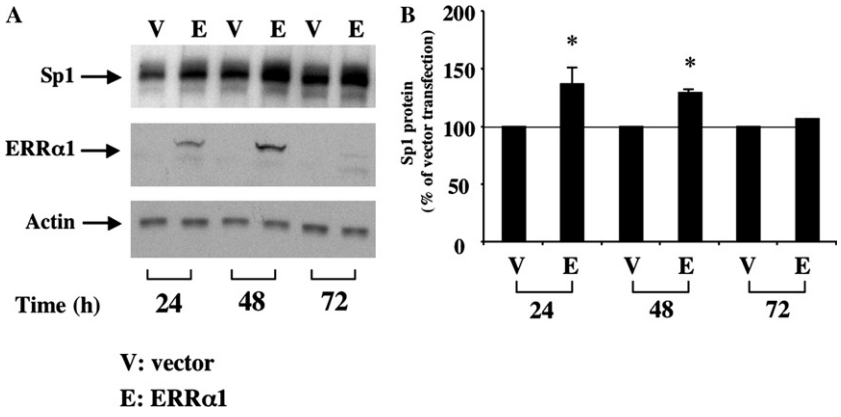

Fig. 3. ERRα1 upregulates endogenous Sp1 protein expression in COS-7 cells. (A) ERRα1 (1–423)/pcDNA3.1(+):E or pcDNA3.1(+):V were transiently transfected into COS-7 cells. Transfected cells were cultured for 24, 48, and 72 h. Twenty microgram of total protein was extracted at each time point and separated by 10% SDS–PAGE. Western blots were performed with anti-Sp1, anti-ERRα1, and anti-actin antibodies. (B) Sp1 band intensity was quantified with NIH-image software and normalized to the actin band intensity. The percent control of Sp1 protein was calculated and further normalized to vector (*$p < 0.05$ vs vector).

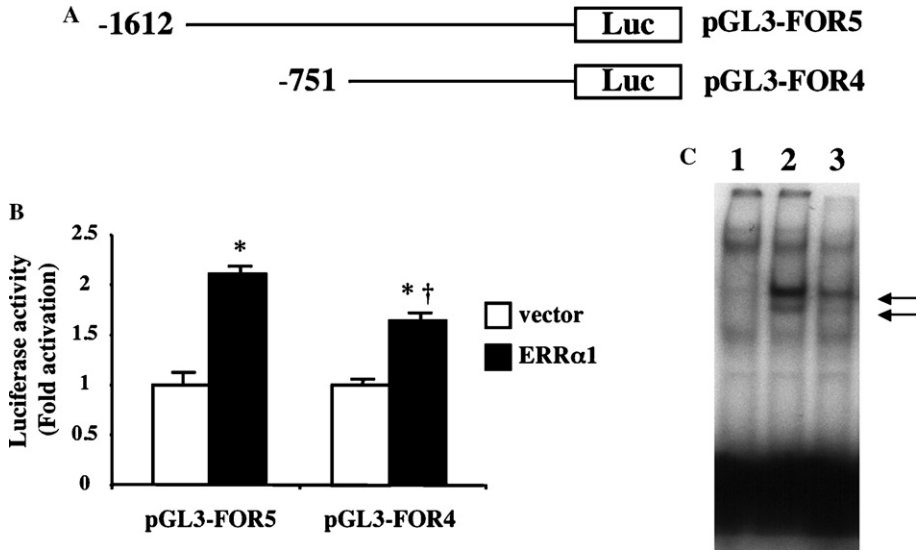

Fig. 4. ERRα1 stimulated Sp1 promoter-dependent luciferase activity and enhanced DNA–protein binding to putative ERRα1 binding site on the human Sp1 promoter. (A) Schematic of the human Sp1 promoter-fused luciferase cDNAs which were used in this study (pGL3-FOR5 and pGL3-FOR4). (B) pGL3-FOR5 or pGL3-FOR4 cDNA were transiently co-transfected with ERRα1 (1–423)/pcDNA3.1(+) (black bar) or pcDNA3.1(+) (white bar) cDNA into COS-7 cells. Cells were cultured for 48 h. Sp1 promoter-dependent luciferase activities were determined and were plotted as fold activation over the individual luciferase activity with co-transfection of vector (*$p < 0.05$ vs vector transfection, $^{\dagger}p < 0.05$ vs the difference of enhancement in pGL3-FOR5 and pGL3-FOR4-dependent luciferase activity by ERRα1). (C) Five micrograms of nuclear extract obtained from pcDNA3.1(+) (lane 1) or ERRα1 (1–423)/pcDNA3.1(+) (lane 2)-transfected COS-7 cells was reacted with oligonucleotide encompassing the putative ERRα1 binding site on the human Sp1 promoter. DNA–protein complexes were confirmed in the presence of 100-fold excess unradiolabeled oligonucleotide (lane 3).

ERRα1 stimulates binding against the putative ERRα1 binding site on human Sp1 promoter

To determine whether the putative ERRα1 binding site in the Sp1 promoter is involved in the ERRα1-mediated upregulation of Sp1 expression, we carried out EMSA using a DNA oligonucleotide containing the putative ERRα1 binding site in the human Sp1 promoter (−1452 to −1427, 5′-AATACTGA**AGGAC ATGACCT**CATCGT-3′). A DNA–protein complex was detected in the nuclear extract obtained from ERRα1-transfected cells as compared with vector transfection (Fig. 4C, lanes 1 and 2). The intensity of this band was reduced in the presence of excess unlabeled oligonucleotide (Fig. 4C, lanes 2 and 3).

The A/B and C domains of ERRα1 are necessary for the activation of Sp1 promoter-dependent luciferase activity

To determine which domains are required for the activation of Sp1 promoter-dependent luciferase activity by ERRα1 transfection, two cDNAs were constructed,

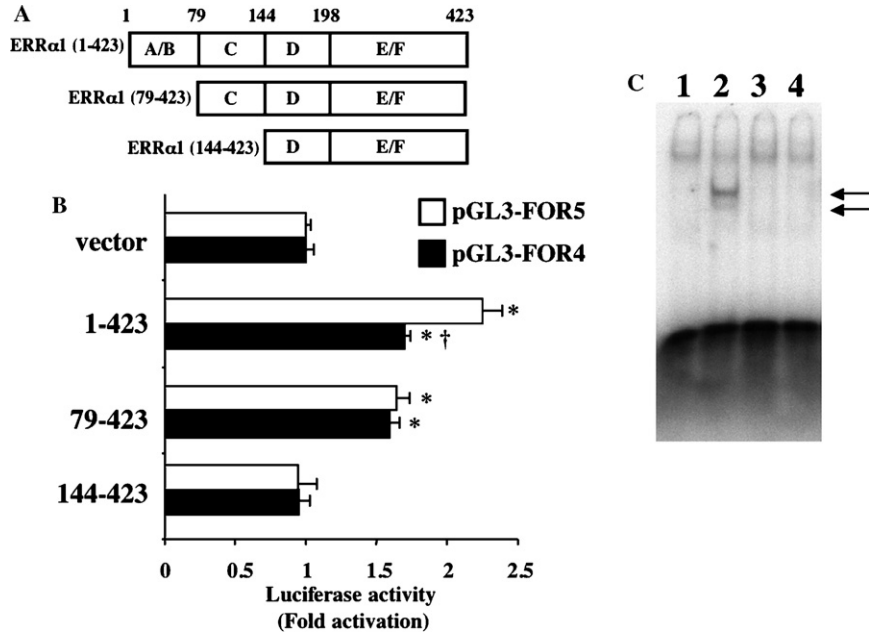

Fig. 5. Sp1 promoter-dependent luciferase activity and DNA–protein binding with truncated ERRα1 in COS-7 cells. (A) Schematic of the truncated ERRα1 proteins which were prepared. (B) pGL3-FOR5 (white bar) or pGL3-FOR4 (black bar) were transiently co-transfected with ERRα1 (1–423), (79–423) or (144–423)/pcDNA3.1(+) cDNAs into COS-7 cells. Cells were cultured for 48 h. Sp1 promoter-dependent luciferase activities were determined and plotted as fold activation over the individual luciferase activity with vector (*$p < 0.05$ vs vector transfection, †$p < 0.05$ vs the difference of enhancement in pGL3-FOR5 and pGL3-FOR4-dependent luciferase activity by ERRα1 (1–423)). (C) Five micrograms of nuclear extract obtained from pcDNA3.1(+) (lane 1), ERRα1 (1–423)/pcDNA3.1(+) (lane 2), ERRα1 (79–423)/pcDNA3.1(+) (lane 3) or ERRα1 (144–423)/pcDNA3.1(+) (lane 4)-transfected COS-7 cells was reacted with oligonucleotide encompassing the putative ERRα1 binding site on the human Sp1 promoter.

an A/B domain deletion mutant: ERRα1 (79–423)/pcDNA3.1(+), and an A/B and C domain deletion mutant: ERRα1 (144–423)/pcDNA3.1(+) (Fig. 5A). The expression of the truncated ERRα1 proteins of the correct molecular weights was confirmed by Western blot (data not shown). As shown in Fig. 5B, ERRα1 (1–423) transfection significantly activated both the full length Sp1–luciferase construct (pGL3-FOR5) and the shorter Sp1–luciferase construct (pGL3-FOR4)-dependent luciferase activity. The deletion of the A/B domain: ERRα1 (79–423) led to a significant decrease in pGL3-FOR5-dependent luciferase activity as compared with ERRα1 (1–423) transfection. Also, additional deletion of C domain: ERRα1 (144–423) caused a further reduction of luciferase activity resulting in a basal activity which was comparable with vector transfection. Although pGL3-FOR4-dependent luciferase activity was enhanced by ERRα1 (1–423) transfection, this ERRα1 (1–423)-enhanced luciferase activation was not reduced by the deletion of the A/B domain.

A/B domain is necessary for ERRα1 binding against putative ERRα1 binding site on human Sp1 promoter

To determine the difference between ERRα1 (1–423)-enhanced pGL3-FOR5 and pGL3-FOR4-dependent luciferase activity, we performed EMSA with the Sp1 promoter oligonucleotide which contained the putative ERRα1 binding site (described in Fig. 4C). As shown in Fig. 5C, a DNA–protein complex was detected with the nuclear extract obtained from ERRα1 (1–423) transfected cells but was absent when cells were transfected with either the A/B deletion construct ERRα1 (79–423) or the A/B and C deletion construct ERRα1 (144–423).

ERα does not activate Sp1 promoter-dependent luciferase activity

To determine whether Sp1 promoter-dependent luciferase activity is activated by ERα, we co-transfected ERα high expression cDNA (ERα/pcDNA3) and the Sp1 promoter–luciferase construct (pGL3-FOR5). To confirm the transient expression of ERα protein, we transfected ERα/pcDNA3 or pcDNA3 into COS-7 cells. Fig. 6A shows that ERα protein could be detected 24 h after transfection with a greater level of expression observed after 48 h. We confirmed that ERE-dependent luciferase activity was induced by 10^{-8} M 17β-estradiol treatment for 24 h. As shown in Fig. 6B, ERE-dependent luciferase activity was activated by ERα transfection in the presence of 17β-estradiol. We then performed a similar experiment to examine Sp1 promoter-dependent luciferase activity. Fig. 6C shows that ERα

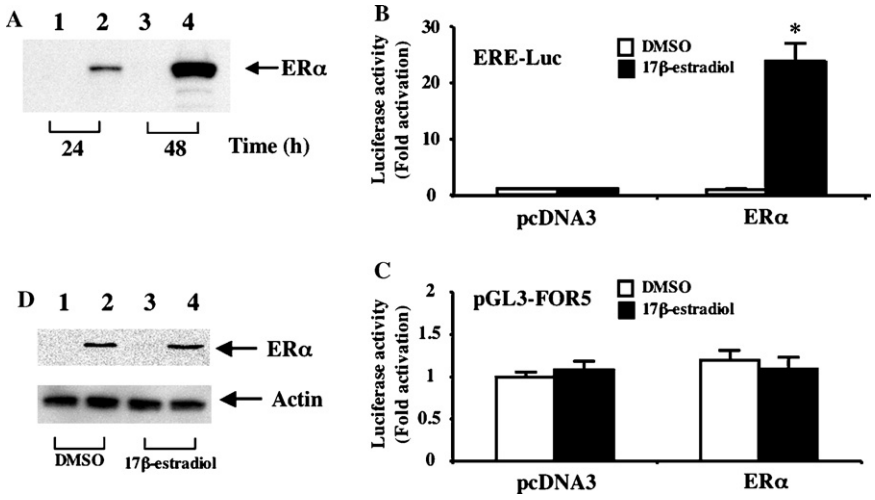

Fig. 6. ERα does not activate Sp1 promoter-dependent luciferase activity. (A) pcDNA3 (lanes 1 and 3) or ERα/pcDNA3 (lanes 2 and 4) was transiently transfected into COS-7 cells. Transfected cells were cultured for 24 and 48 h. Ten micrograms of total protein was extracted and separated by 10% SDS–PAGE. Western blots were performed with anti-ERα monoclonal antibody. ERE-luciferase (B) or pGL3-FOR5 (C) was transiently co-transfected with ERα/pcDNA3 or pcDNA3 into COS-7 cells. Cells were cultured for 24 h and media were changed in the presence (black bar) or absence (DMSO, white bar) of 10^{-8}M 17β-estradiol for 24 h. ERE and Sp1 promoter-dependent luciferase activities were determined and were plotted as fold activation over the individual luciferase activity with vector and in the absence of 17β-estradiol (*$p < 0.05$ vs ERα transfection in the absence of 17β-estradiol). (D) Cell lysates were obtained from the same procedure as (C). Western blots were performed with anti-ERα and anti-actin monoclonal antibodies.

does not activate Sp1 promoter-dependent luciferase activity in the absence or presence of 17β-estradiol. In order to exclude the possibility that the ERα protein was not expressed after co-transfection with pGL3-FOR5, we examined ERα protein expression by Western blot analysis using the cell lysate obtained from ERα/pcDNA3, pGL3-FOR5, and β-galactosidase-transfected cells (Fig. 6D). This confirmed that, despite the expression of ERα, there was no activation of Sp1-dependent luciferase.

Discussion

In our previous study, we have shown that ERRα1 upregulates eNOS expression at the transcriptional level using a human eNOS promoter-fused luciferase reporter assay [10]. In order to understand the exact region that is required for eNOS promoter activation by ERRα1, we mutated the SFRE on the eNOS promoter (−805: 5′-TCAAGCTCT-3′: −796 and −593: 5′-TCAACCACA-3′: −585). However, we could not detect significant inhibition by these mutations [10]. We hypothesized that ERRα1 activates eNOS transcription through certain transcription factors, so we have examined the Sp1 and AP-1 transcription factors, which are known to govern constitutive eNOS expression [21]. In this study, we demonstrate that ERRα1 transfection enhances the formation of an Sp1–DNA complex by EMSA and activates luciferase activity that is controlled by Sp1 consensus binding sites (Figs. 1 and 2). We also determined whether ERRα1 transfection upregulates Sp1 expression. We find that ERRα1 upregulates Sp1 expression, and thus stimulates Sp1 binding activity. It has been shown that ERα enhances the formation of an Sp1–DNA complex by ERα/Sp1 protein interaction and can regulate gene transcription [17–19]. It is also possible that ERRα1 interacts with Sp1, and this interaction causes an enhancement of Sp1 binding activity. We hypothesized that if an ERRα1/Sp1 protein interaction enhances Sp1–DNA complex formation, we would be able to detect a supershift by EMSA, due to the ERRα1/Sp1 interaction (Fig. 1, lanes 1 and 2 of Sp1). However, we could not detect any supershifted band compared with nuclear extracts that were obtained from vector and ERRα1 transfection. It remains to be examined whether ERRα1/Sp1 protein interaction enhances Sp1–DNA complex formation under our experimental conditions.

In this study, we showed that ERRα1 upregulates Sp1 expression at the transcriptional level. In order to know the exact region which is necessary for ERRα1 regulation on the Sp1 promoter, we used two human Sp1 promoter-fused luciferase cDNAs (pGL3-FOR5 and pGL3-FOR4). pGL3-FOR5 and pGL3-FOR4 are Sp1 promoter-fused luciferase cDNAs that include from −1612 to −21 and from −751 to −21 of the human Sp1 promoter region (Fig. 4A). We detected a significant decrease between the luciferase activity obtained from co-transfection with vector and pGL3-FOR5 or pGL3-FOR4 (data not shown). This suggests that constitutive Sp1 expression is regulated by the region from −1612 to −751 on the promoter which is consistent with a previous report using 293T cells [20]. In addition, we

postulated that the required regions for ERRα1 transactivation are located on the Sp1 promoter from −1612 to −751, as we obtained a significant difference in the enhancement of ERRα1 (1–423) transactivation between pGL3-FOR5 and pGL3-FOR4 (Figs. 4B and 5B). We predicted one site between −1444 and −1433 on the Sp1 promoter was important for ERRα1 binding. The DNA sequence from −1444 to −1433 on the Sp1 promoter is 5′-AGGACATGACCT-3′ and is similar to the consensus ERE which is defined as a palindrome of the sequence 5′-AGGTCA-3′. Indeed, we detected DNA–protein complexes by EMSA which show the interaction with the predicted ERRα1 binding region and protein (Figs. 4C and 5C). Although we showed that ERRα1 binds to this predicted region from −1444 and −1433, and enhances Sp1 transcription, ERRα1 can still activate pGL3-FOR4-dependent luciferase which lacks this promoter region. This may be due to the presence of transcription factor binding regions which might be involved in the activation of Sp1 transcription by ERRα1.

The luciferase activity obtained from co-transfection of pGL3-FOR5 and some truncated ERRα1 cDNAs showed that the A/B and/or C domains are required for Sp1 transcription. The truncation of the A/B domain decreased pGL3-FOR5-dependent luciferase activity, however pGL3-FOR4-dependent luciferase activity was not decreased by A/B truncation (Fig. 5B). In addition, nuclear extracts obtained from ERRα1 (79–423)/pcDNA3.1(+)-transfected cells did not show any bands on the EMSA compared with ERRα1 (1–423)/pcDNA3.1(+) (Fig. 5C). In summary, the A/B domain of ERRα1 is required for the binding to the predicted site on the Sp1 promoter and also for activation of Sp1 transcription by ERRα1. However, it is still unknown how the A/B domain regulates the formation of a DNA–protein complex. Although the C domain of ERRα1 activates Sp1 promoter-dependent luciferase shown by the comparison of the ERRα1 (79–423) and ERRα1 (144–423) transfection in Fig. 5B, it remains to be investigated exactly which region on the Sp1 promoter is required for C domain-dependent activation.

It has been shown that Sp1 activity is dependent on several different protein kinases including casein kinase II [22], protein kinase C [23], and p42/44 mitogen-activated protein kinase (MAPK) [24]. Indeed, these protein kinases phosphorylate Sp1 protein and affect binding activity. Furthermore, the estrogen/ERα pathway has been shown to activate some protein kinases. For example, estrogen stimulates p42/44 MAPK through the estrogen receptor after 5–10 min treatment [25] and estrogen/ERα activates phosphoinoside 3-kinase (PI3-kinase) by association with p85α (PI3-kinase regulatory subunit) in a ligand-dependent manner within 30 min [14]. These reports provide another indirect mechanism by which ERRα1 could enhance Sp1 activity by phosphorylation of Sp1 via certain protein kinases in the transfected cells.

Recently, several studies have investigated whether the expression and activation of ERRα and PGC-1 are related during mitochondrial ATP synthesis. Indeed, the expression patterns of ERRα and PGC-1 are very similar, and ERRα is required for PGC-1-induced ATP synthase β and cytochrome *c* expression which are involved in ATP synthesis [26]. Also, ERRα is highly expressed in skeletal muscle and brown fat which are regarded as important tissues for fatty acid β-oxidation [5,7]. Indeed, ERRα1 upregulates MCAD expression, which catalyzes the initial step in mitochondrial fatty acid β-oxidation [6,7]. Although the relationship between the Sp1 transcription factor and mitochondrial ATP synthesis is not clearly understood, ERRα1 may modulate ATP synthesis via Sp1, because Sp1 has been shown to upregulate cytochrome *c* expression [27], and is implicated in the formation of a DNA–protein complex on the MCAD promoter region [28].

ERα is known to bind to ERE that is characterized as the sequence 5′-AGGTCAnnnTGACCT-3′, which forms a homodimer [29,30]. Our data show that ERα transfection-activated ERE-dependent luciferase activity, even though ERα transfection did not activate Sp1 promoter-dependent luciferase activity (Figs. 6B and C). The predicted ERRα1 binding site on the human Sp1 promoter is 5′-AGGACATGACCT-3′, and this DNA sequence displays some similarity to the ERE. However, the 3 bps spacing (nnn) is not included in our predicted ERRα1 binding site and there is one mutation from A to T at position 4. The lack of 3 bps spacing and/or one mutation in the predicted ERRα1 binding site on the human Sp1 promoter might be responsible for the lack of activation of Sp1 promoter-dependent luciferase activity by ERα transfection.

In this study, we show that ERRα1 activates Sp1 activity and upregulates Sp1 expression at the transcriptional level. Also, we found an ERRα1 binding site in the Sp1 promoter region and that binding to this site was regulated by the A/B domain of ERRα1. The upregulation of Sp1 promoter, which could be detected after ERRα1-dependent transactivation, was not detected by ERα-dependent transactivation.

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