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E6-AP facilitates efficient transcription at estrogen responsive promoters through recruitment of chromatin modifiers

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ABSTRACT

E6-AP is a known coactivator of the estrogen receptor alpha (ERα), however the coactivation mechanism of E6-AP is not clear. This work was undertaken to elucidate the coactivation mechanism of E6-AP. In order to examine the role of E6-AP in ERα signaling, we knocked-down the expression of E6-AP and examined the transactivation functions of ERα. Knockdown of E6-AP showed reduced mRNA production of the ERα target genes pS2 and GREB1 suggesting that E6-AP is required for their proper transcription facilitated by ERα. In order to study the mechanism(s) by which E6-AP regulates the transcriptional functions of ERα, we performed chromatin immunoprecipitation (ChIP) assays under E6-AP knockdown conditions. Our ChIP data suggest that knockdown of E6-AP leads to decreased recruitment of the histone acetylase p300 to the ERα target gene pS2 promoter as well as reduced histone modifications at the promoter. Although there was reduced p300 recruitment to the pS2 promoter, loss of p300 did not account fully for the loss of histone acetylation. Taken together our data suggest that E6-AP regulates the transactivation functions of ERα in part by complexing with p300 and other chromatin modifying enzymes at target gene promoters to create a transcriptionally active promoter environment.

1. Introduction

Transcription of DNA into RNA is a tightly controlled process with multiple points of regulation. RNA polymerase II (RNA pol II) transcribes DNA and can be regulated by its access to the promoter. The general steps for transcription have been well characterized. These include chromatin opening, preinitiation complex (PIC) formation, initiation, promoter escape, escape from pausing, elongation, and termination [1]. Genome-wide chromatin immunoprecipitation (ChIP) analysis of RNA pol II has shown 3 distinct gene types divided by RNA pol II occupancy on the gene: (1) no RNA pol II, (2) evenly distributed RNA pol II, and (3) RNA pol II enriched at the 5′ end of genes [2]. pS2 (Trefoil factor 1) is one of the well studied estrogen responsive genes that has very low association of RNA pol II on its promoter at all times but displays cycling of transcription factors and changes in the total recruitment of RNA pol II upon estrogen stimulation [3]. This indicates that access to the promoter is the rate limiting step in transcription of pS2. It has been shown that upon estrogen induction, preformed complexes of coactivators of transcription are recruited to the pS2 promoter along with the estrogen receptor alpha (ERα) to interact with general transcription factors (GTFs) and modify chromatin structure to induce gene transcription [4].

ERα is a ligand activated transcription factor, which mediates most of the effects of estrogen signaling within cells. ERα is a member of the nuclear hormone receptor superfamily and has a well characterized domain structure. There are 6 domains within ER designated A–F. A/B region contains the activation 1 domain, C is the DNA binding domain, D is the hinge region, E is the activation 2 domain which contains the ligand binding domain (LBD), and F is a partially characterized domain thought to be important in receptor dimerization [5]. Upon estrogen binding, the LBD undergoes a conformational change to create a hydrophobic cleft supported by helices 3, 4, and 12. This cleft allows the LXXLL motif found in coactivators to interact with ERα [6]. ERα coactivators are proteins, which usually have enzymatic functions such as histone acetyl transferase (HAT) and methyltransferase activity, that facilitate chromatin remodeling to destabilize DNA–histone interactions, or provide structural support for the recruitment of RNA pol II with other components of the basic transcription machinery and/or other coactivators [7]. The ultimate effect of coactivators is to increase ERα-mediated transcription.

E6-associated protein (E6-AP) acts as both an E3 ubiquitin ligase and a coactivator of ERα [8]. E6-AP was first characterized as an E6 interacting protein in Human Papillomavirus that mediates

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p53 degradation during infection [9]. It was later discovered that E6-AP plays a critical role in Angelman Syndrome and a role in Prader–Willi Syndrome in which UBE3A, the gene encoding for E6-AP, is either mutated, silenced or deleted [10]. Interestingly in Prader–Willi patients, puberty is either delayed or incomplete suggesting a role for E6-AP in sexual development. Indeed in mouse knockout models of E6-AP, female mice are sub-fertile [11]. E6-AP is thought to play a role in estrogen-mediated degradation of ERα as a mechanism of coactivation [8], but the exact mechanism of E6-AP coactivation function remains unclear. In this study we show that knock-down of E6-AP leads to reduced p300 recruitment and reduced chromatin modifications at the pS2 promoter site indicating that the coactivation mechanism of E6-AP involves in part recruitment of chromatin modifying enzymes to create an active promoter environment.

2. Materials and methods

2.1.1. Cell culture and reagents

MCF-7 cells were maintained in DMEM high glucose (Invitrogen, Carlsbad, CA Cat#10569) with 5% FBS (Atlanta Biological, Lawrenceville, GA), 100 μg/mL penicillin/streptomycin and 5% CO_2. T47D cells were maintained in RPMI (Invitrogen Cat#61870) with 5% FBS, 100 μg/mL penicillin/streptomycin and 5% CO_2. 17β-Estradiol/estrogen (Sigma, MO) was prepared in ethanol.

2.1.2. siRNA knockdown of E6-AP

1×10^6 MCF-7 cells were plated in 10 mL on 100 mm dishes with 5% charcoal stripped fetal bovine serum (cFBS) in phenol red free DMEM (Invitrogen Cat#21063) for 24 h. Cells were then transfected with 200 pmols of siRNA On-Target plus smart pool (Dharmacon, Chicago, IL) or control siRNA Scramble using Lipofectamine RNAi max (Invitrogen) according to the manufacturer's protocols. 48 h after transfection, hormone treatments were begun using ethanol vehicle control or 10^{-8} M 17β-estradiol. MCF-7 cells were treated with estrogen as indicated.

2.1.3. ChIP

ChIP was similar to as previously described [12]. Cells were grown in cFBS in 100 mm plates as in siRNA knockdown described above. 24 h after siRNA treatment, media was changed (5% cFBS). After another 48 h (72 h after siRNA treatment) cells were treated with estrogen for various times. Cells were then fixed with 1% formaldehyde (in PBS) for 10 min, washed in ice cold phosphate-buffered saline (PBS) for 5 min, and then quenched with glycine stop buffer (125 mM Glycine in PBS) for 5 min. Cells were subsequently washed in ice cold PBS for 5 min. Cells were then scraped in PBS containing phenylmethylsulfonyl fluoride (PMSF), spun, and resuspended in lysis buffer (50 mM HEPES pH 8.0, 1 mM EDTA, 140 mM NaCl, 10% glycerol, 0.5% NP-40, and 0.25% Triton X-100) to sit on ice for 30 min. Lysate was then homogenized with a Type-B Dounce homogenizer using 15 strokes per sample. Nuclear fraction was then collected by pelleting and resuspended in 600 μL of sonication buffer (10 mM Tris pH 8.0, 1 mM EDTA, 140 mM NaCl, 1% SDS, 0.1% sodium deoxycholate, and 1% Triton X-100). Cells were then sonicated using a Misonix S-4000 sonicator for 10 s at 20% power (~55 J). 10 μL of the sheared DNA was aliquoted out and considered input DNA. For ChIP, 100 μL of sheared DNA was diluted 10 fold in buffer Y (16.7 mM Tris pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, and 1.2 mM EDTA). Diluted sheared DNA was precleared with 2 μg of appropriate IgG antibody and salmon sperm coated DNA Agarose Beads (Millipore) for 1 h at 4 °C. Beads were spun at 0.6 rcf for 1 min and supernatant was moved to a fresh tube. 2 μg of specific antibody was then used for overnight IP at 4 °C. Salmon sperm coated DNA Agarose Beads were then added for 1.5 h incubation. Beads were spun at 0.6 rcf for 1 min. Beads were washed once in TSEI (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl), once in TSEII (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 500 mM NaCl), once in TSEIII (0.5 M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.1, 150 mM NaCl) and then twice in TE (1 mM EDTA, 10 mM Tris pH 8.1) for 5 min each. DNA was then eluted twice using Elution Buffer (1% SDS and 0.1 M NaHCO3) for 15 min each elution, 75 μL of buffer each time. 10 μL of input DNA was diluted 1:10 with buffer Y. 150 μL elutions and input DNA were then reverse cross-linked by adding NaCl to a final concentration 200 mM and incubating for 5 h or overnight at 65 °C. Proteins were removed by bringing elutions to a final concentration of 10 mM EDTA and 40 mM Tris pH 6.8 with 1.25 units of Proteinase K for 1.5 h at 45 °C. DNA from elutions was then purified using a Qiagen pcr purification kit. DNA was used directly in qPCR reactions. Antibodies used were pRNA pol II (Abcam 5095-100), H3K14 (Abcam Ep964Y), p300 (C-20 Invitrogen) E6-AP (N-20 Invitrogen), and ERα (HC20).

2.1.4. Lysis

Cells were washed once with PBS. RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Sodium Deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM EDTA pH 8.0, protease inhibitor cocktail (Sigma)) or TMI buffer (150 mM Tris–HCl, pH 7.5, 150 mM NaCl, 20 mM NaMoO4, 0.09% Igepal (NP-40 substitute), protease inhibitor cocktail (Sigma)) was added to cells and scraped. Cell lysate was spun at 16.1 rcf, 4 °C for 10 min and supernatant was frozen at −80 °C for Western analysis.

2.1.5. Western blot

50 μg of total protein was run on a 10% SDS-PAGE at 100 V and subsequently transferred to a nitro-cellulose membrane (0.2 μm pore) using 100 V for 2 h at 4 °C. Membranes were blocked using 5% milk (50 mM Tris pH 7.5 and 150 mM NaCl) for at least 1 h. Membranes were probed with primary antibody (2.5% milk, 25 mM Tris pH 7.5 and 75 mM NaCl) for at least an hour, washed 3 times in TBS-T, 5 min each wash, and probed with horseradish peroxidase (HRP) secondary antibody for 1 h. Membranes were then washed 3 times in TBS-T and chemiluminescence was added. AC-15 Beta Actin antibody is from Sigma. ERα (HC20, F-10), E6-AP (EE7), and p300 (C-4) antibodies are from Santa Cruz. E6-AP (446 and 447) is from Bethyl Laboratories.

2.1.6. cDNA production

MCF-7 cells grown in 6-well plates were washed once with PBS and 350 μL of buffer RLT (Qiagen) was added to each well. Cells were then frozen at −80 °C for later processing. RNA was isolated from 6-well plates following the manufacturer's protocol for Qiagen RNeasy mini kit. Lysate was homogenized by either passing the lysate through a QIShredder (Qiagen) or pipeting up and down with a 1 mL pipett tip (10×). 2 μg of total RNA was used to create cDNA using Fermentas Maxima cDNA first strand cDNA synthesis kit according to the manufacturer's protocol.

2.1.7. Real-time PCR (qPCR) for mRNA

For mRNA qPCR, 4% of the cDNA was used in 12.5 μL reactions for the real-time PCR reactions. Cycle threshold (CT) values were normalized to 36B4 mRNA levels [13]. Total primer concentration was 200 μM for forward and reverse primers. SYBR Green mastermix (2×) from Invitrogen was used in 12.5 μL total reaction volumes. Real-time PCR was carried out on a LightCycler 480 (Roche). CT val-