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Interaction of positive coactivator 4 with histone 3.3 protein is essential for transcriptional activation of the luteinizing hormone receptor gene

Peng Zhao[1], Raghuveer Kavarthapu[1], Rajakumar Anbazhagan[1], Mingjuan Liao, Maria L. Dufau*

Section on Molecular Endocrinology, Division of Developmental Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

The luteinizing hormone receptor (LHR) is essential for sexual development and reproduction in mammals. We have established that Sp1 has a central role in derepression of LHR gene transcription induced by Trichostatin A (TSA) in MCF7 cells. Moreover, the co-activator PC4 which associates directly with Sp1 at the LHR promoter is essential for TSA-mediated LHR transcription. This study explores interactions of PC4 with histone proteins, which presumably triggers chromatin modifications during LHR transcriptional activation. TSA treatment of MCF7 cells expressing PC4-Flag protein induces acetylation of histone 3 (H3) and immunoprecipitation (IP) studies revealed its interaction with PC4-Flag protein. MS/MS analysis of the protein complex obtained after IP from TSA treated samples detected H3.3 acetylated at K9, K14, K18, K23 and K27 as a PC4 interacting protein. The association of PC4 with H3.3 was corroborated by IP and re-ChIP using H3.3 antibody. Similarly, IP and re-ChIP showed association of PC4 with H3 acetylated protein. Knockdown of PC4 in MCF7 cells reduced H3.3 enrichment, H3 acetylation at the Lys sites and LHR promoter activity in TSA treated cells despite an increase in H3 and H3.3 protein induced by TSA, linking PC4 to H3 acetylation and LHR transcription. Depletion of H3.3 A/B in MCF7 cells impair chromatin accessibility and enrichment of Pol II and TFIIB at the LHR promoter and its activation, resulting in marked reduction of LHR gene expression. Together, these findings point to the critical role of PC4 and its association with acetylated H3.3 in TSA-induced LHR gene transcription.

1. Introduction

The luteinizing hormone receptor (LHR) is a member of the G-protein-coupled receptor family, which is primarily expressed on the surface of gonadal cells. This receptor mediates responses to luteinizing hormone (LH) signals, which regulate cyclic changes in the structure/function of the ovary, and the development and function of Leydig cells in the testis [1–4]. In both types of reproductive organs, cyclic AMP-mediated activity of LH/LHR supports regulation of steroid hormone production by steroidogenic enzymes, which is essential to maintenance of reproductive functions, including ovulation and spermatogenesis [3,4]. LHR is also expressed in non-gonadal cells, tumor tissue, and cancer cells [5,6].

Stable cultures of cancer cells provide major advantages over transient cultures of gonadal cells and have permitted in-depth exploration of basic aspects of transcription and the identification of complex regulatory systems [7]. Early studies in JAR choriocarcinoma and MCF7 cancer cells, demonstrated that *LHR* transcriptional expression is repressed under basal conditions because of histone hypoacetylation [6]. Subsequent studies using the histone deacetylase inhibitor, trichostatin A (TSA), which promotes histone acetylation, derepression of the *LHR* gene, and activation of its transcription in these cells, revealed its regulation by complex and diverse networks, in which coordination and interactions between regulatory effectors are essential for silencing and activation of *LHR* expression [6,8–11].

The proximal Sp1 site in the 176 bp *LHR* promoter recruits histone (H) deacetylases and the Sin3A corepressor complex, which contribute to the silencing of *LHR* transcription [8]. Site-specific acetylation/methylation-induced phosphatase release serve as an on-switch for Sp1 phosphorylation at Ser641 by PI3K/PKCζ [8,9]. These processes cause release of the repressor, pRb homologue 107, from Sp1, enrichment of TFIIB and Pol II, and transcriptional activation [10]. Maximal derepression of the gene is dependent on DNA demethylation of the promoter, H3/H4 acetylation, and HDAC/Sin3 A release [6,8]. In further studies, our laboratory demonstrated that positive cofactor 4 (PC4) has an important role in assembly of the preinitiation complex in

* Corresponding author at: Section on Molecular Endocrinology, NICHD, National Institutes of Health, Bethesda, MD 20892, USA.
E-mail address: dufaum@mail.nih.gov (M.L. Dufau).
[1] These authors contributed equally to this study.

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TSA–mediated *LHR* transcription [11]; PC4 is recruited by Sp1 following TSA treatment and acts as its coactivator. The coactivator domain of PC4 (amino acids (aa) 22–91) and the DNA-binding domain of Sp1 are required for PC4/Sp1 interaction [11]. Only the non-phosphorylated form of PC4 interacts directly with Sp1, and the interaction is inhibited by PC4 phosphorylation. PC4 does not participate in TSA-meditated release of phosphatases from Sp1, Sp1 phosphorylation, or release of repressor complexes. Although TFIIB enrichment is dependent on PC4, we ruled out TFIIB as its direct target in the activation process [11].

In this study, we demonstrate that TSA induces acetylation of a PC4-interacting protein, identified as acetylated H3.3. We also identified the sites of acetylation of the H3.3 histone variant by MS/MS analysis, its interaction with PC4, and its presence in a complex associated with chromatin in the promoter region. The H3.3-PC4 interaction is essential for TSA-induced transcriptional activation and expression of the *LHR* gene.

2. Materials and methods

2.1. Reagents and antibodies

Trichostatin A (TSA) was purchased from Calbiochem. The antibody against PC4 (#PA-117-01) was obtained from ProteinOne, Rockville, MD, and those for β-actin (#SC69879) and Pan-acetyl C2 (#SC8649) from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against H3 (#4499S), H3K9-Ac (#9649S), H3K14-Ac (#7227S), H3K18-Ac (#13998S), H3K23-Ac (#8848S), H3K27-Ac (#8173S), H3K36-Ac (#11885), H2A (#2778S), Histone H2A-Ac, Histone H2B (#8135S), Histone H2B-Ac, Histone H4 (#2592), Histone H4-Ac-Ser1/Lys5/Lys/Lys12 (#SC377520), and GST (#2625S) were purchase from Cell Signaling Technology Inc., Danvers, MA. H3.3 antibody (monoclonal) was obtained from Abcam (Cambridge, MA) (#ab176840). Antibodies against TFIIB (#SC271736) and Pol II (#05-623) were from Santa Cruz Biotechnology and Millipore (Burlington, MA), respectively. FLAG antibody (#8146S), anti-FLAG M2 Affinity gel, and Mouse IgG agarose were purchased from Sigma-Aldrich (St. Louis, MO) (#A2220). Recombinant H3, H4, H3.3, and H3-H4 tetramer were obtained from New England Biolabs (Ipswich, MA) and recombinant H3.3-H4 tetramer was from Sigma-Aldrich.

2.2. Expression vectors and cell culture

The reporter gene construct containing the *LHR* promoter was generated by cloning the human *LHR* gene promoter region (−176 to +1) into the *Sac*I/*Bgl*II sites of the pGL2 basic vector [12]. pCMV6-PC4 was purchased from Origene (Rockville, MD) and used as PCR template for generation of constructs expressing PC4-Flag protein in MCF7 cells. p3XFLAG-PC4 vector was created by inserting PCR-amplified *PC4* cDNA into the *Eco*RI and *Kpn*I sites of the p3XFLAG-CMV-7.1 vector (Sigma) [11]. MCF7-A2 cells, kindly provided by Dr. Erica Berleth (C. Roswell Park Cancer Institute, Buffalo, NY) [13], were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Invitrogen). All studies were conducted using cells of passages 6–8. Cells plated in 10-mm culture dishes, were transfected with p3XFLAG-PC4 vector (10 μg) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Transfection of an equal amount of empty p3XFLAG vector was used as a negative control.

2.3. siRNA analysis

Validated siRNAs designed to knock-down the endogenous expression of PC4 and scrambled negative control siRNA were purchased from Ambion (Austin, TX). Transfections of siRNA into MCF7 cells were performed using siPORT Neo FX reagent (Invitrogen), as previously described [11]. Validated siRNAs from Ambion were used to knock-down H3.3A and H3.3B. The siRNA sequences were as follows: 5′-UUAAAGUCCUGAGCAAUUUCT-3′ (H3.3A siRNA ID# S6422) and 5′-UAUGAGAACAAGUGCAGUCAG-3′ (H3.3B siRNA ID# S6424). After 24 h of transfection, cells were grown in RPMI 1640 medium for additional 48 h before harvesting. For reporter gene assays, the *LHR* gene promoter and reporter gene were introduced into cells using Lipofectamine and Plus Reagent at 24 h post-transfection of siRNA, and luciferase activity was determined 40 h later. TSA (500 ng/ml) or vehicle were added to cells 16 h prior to harvest.

2.4. RNA isolation and real-time RT-PCR

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA) followed by treatment with DNase I (Invitrogen), as previously described (11). Total RNA was reverse-transcribed with random primers for synthesis of first strand cDNA using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA). Relative levels of *LHR* mRNA were determined by real-time PCR using SYBR-Green Master Mix in an ABI 7500 sequence detection system, as previously described [14]. Relative *LHR* mRNA levels were calculated using the comparative C_T method, with human β-actin as an internal control. The *LHR* primer sequences were: 5′-TCTACACCCTCACCG TCATCACTC-3′ (forward) and 5′-AGCCATCCTCCAAGCATAATCA-3′ (reverse).

2.5. Immunoprecipitation (IP)

MCF7 cells transfected with p3XFLAG-PC4 or empty vector were treated with or without TSA (500 ng/ml) for 16 h. Cell lysates were then extracted using IP lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100; pH 7.4) in the presence of protease inhibitor. The Bradford protein assay was used to determine protein concentration. Proteins (1 mg per reaction) were then pre-cleared by incubation with 40 μl mouse-IgG agarose for 3 h with end-to-end rotation at 4 °C. Supernatants were collected and immunoprecipitated using 40 μl anti-FLAG M2 affinity gel overnight. Agarose/protein complexes were washed four times with IP lysis buffer and once with TE buffer. Sample buffer (2 × 40 μl) was used to elute protein complexes from affinity agarose. After heating at 70 °C for 10 min, samples were subjected to western blot analyses, which were performed as described previously [11]. In experiments where immunoprecipitated proteins were examined using gel staining, IP samples eluted from several IP reactions were concentrated using an Amicon Ultra filter (Millipore) prior to loading on tris-glycine gels.

2.6. GST-pull down assay

The GST-PC4 vector was generated by inserting PCR amplified *PC4* cDNA into the *Eco*RI and *Xho*I sites of the pET-41a (+) vector (Novagen). GST-tagged protein and GST-PC4 protein were expressed in *Escherichia coli* strain Rosetta™ 2 (DE3) (ThermoFisher Scientific). After culture for 16 h at 37 °C, cells were diluted 1:50 and cultured for 3 h at 37 °C, followed by incubation with 0.5 mM IPTG for 6 h at 37 °C. Cultures were harvested and lysed using GST-pull-down lysis buffer (ThermoFisher Scientific), supernatants collected, and the expression of GST-PC4 confirmed by western blotting using GST antibody. GST-pull down assays were performed using a GST Protein Interaction Pull-Down kit (ThermoFisher Scientific), according to the manufacturer's protocol. Briefly, bacterially expressed GST-tagged or GST-PC4 proteins immobilized on GST-agarose resin were used as bait proteins. The resin with immobilized GST-PC4 was incubated overnight with total protein extracts (as prey) isolated from MCF7 cells treated with and without TSA for 16 h. Beads were washed five times with wash buffer to remove unbound proteins and eluted with elution buffer containing 10 mM glutathione to collect bound proteins. The eluted samples were resolved by SDS-PAGE for western blot analysis using H3, H3.3, and H4