ARTICLE
Journal of Cellular Biochemistry 105:365–369 (2008)

Journal of Cellular Biochemistry

Association of Sp3 and Estrogen Receptor α With the Transcriptionally Active Trefoil Factor 1 Promoter in MCF-7 Breast Cancer Cells

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ABSTRACT

To further explore the role of Sp1 and Sp3 in the estrogen regulated TFF1 gene transcription, chromatin immunoprecipitation (ChIP) assay was used to determine the association of estrogen receptor α (ERα), Sp1 and Sp3 with the endogenous trefoil factor 1 (TFF1) gene promoter in MCF-7 breast cancer cells. ERα and serine 5 phosphorylated RNA polymerase II, the form of RNA polymerase II associated with transcription initiation, were recruited to the TFF1 gene promoter following estrogen addition to MCF-7 cells cultured under estrogen deplete conditions. Both Sp1 and Sp3 were bound to the TFF1 gene promoter before and after estrogen treatment. Using the re-ChIP assay, we demonstrate that either Sp1 or Sp3 but not both bind to a TFF1 promoter. The co-occupancy of ERα and Sp1 on TFF1 promoter remains at similar level with and without estrogen, while that of ERα and Sp3 increased in the presence of estrogen. Further, we observed increased co-occupancy of Sp3 and serine 5 phosphorylated RNA polymerase II on the TFF1 promoter after estrogen treatment of cells. Taken together, these results provide evidence that Sp3 and ERα are involved in the estrogen induced transcription of the TFF1 gene. J. Cell. Biochem. 105: 365–369, 2008.

KEY WORDS: Sp1; Sp3; ESTROGEN RECEPTOR; TREFOIL FACTOR 1 GENE; CHROMATIN IMMUNOPRECIPITATION

Sp1 and Sp3 are ubiquitously expressed transcription factors in mammalian cells, with Sp1 often being expressed at a higher level than Sp3. Although Sp3 is structurally similar to Sp1, there are significant differences between these two factors as illustrated in knock out studies and studies investigating their transcriptional roles [Sapetschnig et al., 2004]. Sp1 typically functions as an activator. In contrast Sp3 functions as a transcriptional activator or repressor, pending upon several parameters, including promoter context and chromatin structure [Sapetschnig et al., 2004]. Sp1 and Sp3 also differ in their ability to form multimers, with Sp1, but not Sp3, capable of forming multimers [Yu et al., 2003]. Recently, we demonstrated that Sp1 and Sp3 occupy different subnuclear sites in MCF-7 breast cancer cells [He et al., 2005].

The estrogen responsive elements (EREs) of genes expressed in human breast cancer cells often have an ERE or half site ERE positioned next to a Sp binding site (ERE (½) $(N)_x$ Sp1) [Abdelrahim et al., 2002]. Regulatory regions of cathepsin D, c-*fos*, and c-*myc* genes have such EREs [Dubik and Shiu, 1992; Abdelrahim et al., 2002]. Chromatin immunoprecipitation (ChIP) assays have demonstrated that estradiol addition to MCF-7 breast cancer cells results in the association of Sp1 and estrogen receptor α (ERα) with the cathepsin D and cyclin D1 promoters [Castro-Rivera et al., 2001]. The estrogen responsive trefoil factor 1 (TFF1) gene codes for a cysteine rich protein that stimulates angiogenesis and the migration of breast tumor cells [Prest et al., 2002; Rodrigues et al., 2003]. In analyses of the TFF1 promoter, we identified an Sp1/Sp3 binding site upstream of an imperfect ERE [Sun et al., 2005]. Mutation of the Sp site upstream of the ERE reduced estrogen responsiveness and prevented binding of Sp1 and Sp3. We applied the ChIP assay to show that following the addition of estradiol, ERα, Sp3 and Sp1 were bound to the TFF1 promoter. Our results suggested that Sp3 and not Sp1 was preferentially occupied the estrogen induced TFF1 promoter. This result was surprising as the level of Sp1 is approximately threefold greater than that in MCF-7 breast cancer cells [Sun et al., 2002].

In this study we investigated the contribution of Sp1 and Sp3 to the estrogen-induced transcription of the TFF1 promoter. Our previous studies showing that Sp1 does not form complexes with Sp3 [Sun et al., 2002] lead us to propose that either Sp1 or Sp3 but not both would occupy the TFF1 promoter. To find out whether Sp3

Grant sponsor: CancerCare Manitoba Foundation, Inc.; Grant sponsor: National Cancer Institute of Canada (funds from the Canadian Cancer Society); Grant sponsor: Canada Research Chair.

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Received 6 May 2008; Accepted 8 May 2008 • DOI 10.1002/jcb.21832 • 2008 Wiley-Liss, Inc.

Published online 9 June 2008 in Wiley InterScience (www.interscience.wiley.com).

was supporting transcription rather than acting as a repressor, we determined whether the estrogen induced TFF1 promoter was co-occupied with Sp3 and ER and whether the Sp3 occupied promoter was associated with RNA polymerase II. Our results provide evidence that Sp3 with ERα supports the transcriptional activity of the estrogen induced TFF1 promoter.

MATERIALS AND METHODS

CELL CULTURE

Human breast cancer cell lines MCF-7 (ER positive and estrogen dependent) and MDA-MB-231 (ER negative and estrogen independent) were cultured in DMEM (GIBCO) medium supplemented with 8% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.3% glucose. MCF-7 cells were cultured under estrogen deplete conditions by growing cells in estrogen-depleted phenol red-free Dulbecco's modified Eagle's medium with 2% dextran-charcoal stripped fetal bovine serum, supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), and 0.3% glucose. The cells were treated with 10 nM 17β-estradiol (E2) for required times. For time course ChIP study and re-ChIP assays, the cells were treated with 2.5 μM α-amanitin for two hours and then exposed the cells 10 nM E2 for up to 45 min with 15 min intervals. All the cells were grown in 37°C humidified incubator with 5% CO_2.

CHROMATIN IMMUNOPRECIPITATION (CHIP) AND reChIP ASSAYS

ChIP and reChIP assays were performed as described previously [Hutchison, 2002; Spencer et al., 2003; He et al., 2005]. 18 A_{260} units of sonicated cell lysate were used for each ChIP assay and 30 A_{260} units were used for each reChIP assay. Anti-Sp1 (Upstate) and anti-Sp3 polyclonal antibodies (Santa Cruz, CA), anti-ERα mouse monoclonal antibody (Novo Castra, UK), anti-serine 5 phosphorylated RNA polymerase II (Abcam) antibodies and rabbit preimmune antibodies (Santa Cruz Biotechnology) were used in ChIP and reChIP assays. DNA fragments from the immunoprecipitated and input fractions were analyzed by semi-quantitative PCR either using TFF1 promoter primers (set 1) (For-, 5′-GAC GGA ATG GGC TTC ATG AGC-3′; Rev-, 5′-GAT AAC ATT TGC CTA AGG AGG-3′) to amplify a 385-bp fragment, or using primers to TFF1 intron A–exon 2 were 5′-CTG GGG CAC CTT GCA TTT TCC-3′ and 3′-GGG GGG CCA CTG TAC ACG TC-5′ to amplify a 229-bp fragment. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The PCR product was sequenced to confirm the fidelity of the PCR product. PCR reactions were consistently monitored to ensure linearity.

We applied quantitative real-time PCR to quantitate the PCR products. The quantitative real-time PCR used SYBR green and the PCR primers listed below for both the inputs and ChIP DNAs. TFF1 promoter primers were 5′-GTG AGC CAC TGT TGT CAG GCC AAG C-3′ (forward) and 5′-CCC ATG GGA GTC TCC TCC AAC CTG A-3′ (reverse) to amplify a 150-bp fragment. The amount of ChIP DNA PCR product per A_{260} was divided by that of the input to calculate % of input. All the PCR products were sequenced for sequence confirmation.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) was done as previously described [Espino et al., 2006]. Briefly, RNA from MCF-7 cells treated with or without 10 nM E2 was isolated using the Qiagen RNeasy Kit (Qiagen, CA). cDNA was synthesized using MMLV Reverse Transcriptase (Invitrogen) and PCR was done with Platinum Taq (Invitrogen). The cDNA template amount was adjusted to ensure linearity. The appropriate primers to detect the expression of TFF1 and GAPDH were listed as below. The primers to TFF1 intron A–exon 2 were 5′-CTG GGG CAC CTT GCA TTT TCC-3′ and 5′-CGG GGG GCC ACT GTA CAC GTC-3′, to GAPDH exon 7 were 5′-CCA GGA AAT GAG CTT GAC AAA GTG-3′ and 5′-AAG GTC ATC CCT GAG CTG AAC GGG-3′. The PCR products were resolved electrophoretically on a 1.5% (w/v) agarose gel and stained with ethidium bromide.

RESULTS

The TFF1 promoter has a Sp1 site positioned upstream of an imperfect ERE (Fig. 1A). To synchronize the TFF1 promoter, estrogen-dependent MCF-7 cells were cultured in the absence of E2 for 3 days followed by incubation with 2.5 μM α-amanitin for two hours to synchronize the promoters [Shang et al., 2000; Metivier et al., 2003]. Following rapid removal of α-amanitin, the cells were exposed to 10 nM E2 for up to 45 min. Immunoblot analyses of Sp1, Sp3 and ERα revealed that there was no change with the levels of these proteins (data not shown). To monitor the expression of the TFF1 gene following the addition of estrogens, RT-PCR was conducted using the housekeeping gene GAPDH as a reference. Increased levels of TFF1 mRNA were detected after 15 min of E2 addition (Fig. 1B).

Following the recruitment RNA polymerase II to a promoter, the enzyme goes through a series of phosphorylation, with phosphorylation at serine 5 showing that the RNA polymerase II has engaged in initiation [Xie et al., 2006]. The occupancy of the initiated form of RNA polymerase II on the TFF1 promoter was determined using the ChIP assay with antibodies against serine 5 phosphorylated RNA polymerase II. Figure 1C shows that following the addition of E2, serine 5 phosphorylated RNA polymerase II, was associated with the TFF1 promoter. No association of RNA polymerase II with the TFF1 promoter was detected in MDA-MB-231 cells in which the TFF1 gene is transcriptionally inactive (data not shown).

TEMPORAL LOADING OF Sp1, Sp3, AND ERα ONTO THE ESTRADIOL STIMULATED TFF1 PROMOTER

To investigate the temporal occupancy of ERα, Sp1, and Sp3 onto the endogenous estrogen induced TFF1 gene, we carried out the ChIP assay with antibodies against ERα, Sp1, Sp3 and rabbit preimmune IgG (as a negative control). The input and ChIP DNA was analyzed with primer sets to the promoter and to the intron A–exon 2 region of the TFF1 gene (see Fig. 1A). As shown in Figure 2A, E2 induced a dramatic increase in the occupancy by ERα on the TFF1 promoter following 15 min E2 addition. A decline in ERα occupancy was

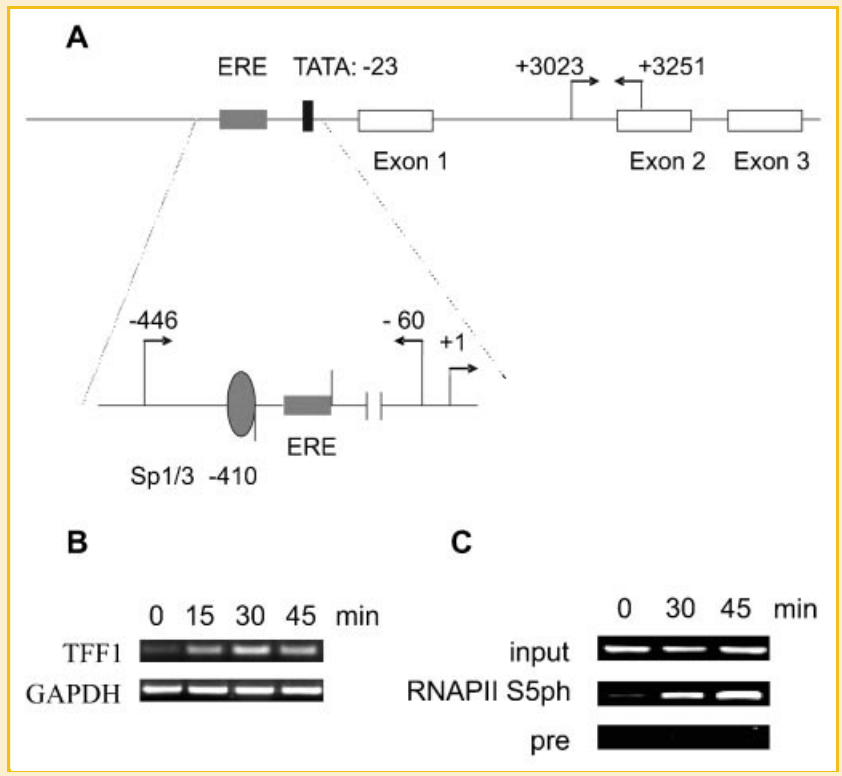

Fig. 1. Estrogen induced expression of TFF1 gene. A: Map of the TFF1 gene. Exons 1–3 are represented by open rectangles. More detailed diagram of the promoter region is shown in the lower portion of the figure. Positions of several regulatory regions in respect to the start site for TFF1 promoter are shown in black rectangles. TATA, TATA box; Sp1, Sp1 and Sp3 cognate element; ERE, estrogen responsive element. B: MCF-7 cells cultured under estrogen- and serum-deprived conditions were treated for 15, 30, 45 min with 10 nmol/L E2. Cells were harvested at the indicated time and RNA was extracted for further RT-PCR analysis with primers corresponding to TFF1 intron A–exon 2 and GAPDH exon VII as loading control. C: Occupancy of the TFF1 promoter by serine 5 phosphorylated RNA polymerase II (RNAP II S5ph) and rabbit preimmune antibodies (pre) (negative control) at different times as measured by the ChIP assay. Input and ChIP DNA were analyzed by primers to the TFF1 promoter.

consistently noted at 45 min. The occupancy of Sp1 on the TFF1 promoter remained unaltered before and after E2 addition, with a slight but reproducible increase observed at 30 min. Sp3 association was also observed with the TFF1 promoter before and after E2 addition. However, a more pronounced increase in TFF1 promoter occupancy was observed for Sp3 compared to Sp1 at 30 min. As a control, we demonstrated that the TFF1 intron A–exon 2 region was not associated with Sp1, Sp3, or ERα (Fig. 2A). Further, in control ChIP assays with rabbit preimmune IgG, minimal or no TFF1 promoter or intron A–exon 2 sequences were detected.

The amount of TFF1 promoter immunoprecipitated with anti-Sp1, Sp3 and ERα antibodies was quantified and presented as % of input DNA (Fig. 2B). In the presence of estradiol, ERα loading steadily increased, peaking at 30 min and then slightly decreasing at 45 min. Following the addition of estradiol, Sp3 association with the TFF1 promoter varied, reaching a maximum at 30 min. In contrast, Sp1 association with the TFF1 promoter was marginally altered.

ERα CO-OCCUPY THE TFF1 PROMOTER WITH Sp3 AFTER ESTROGEN ADDITION TO CELLS

Our previous studies demonstrated that Sp1 and Sp3 have distinct nuclear locations and do not form complexes together [Sun et al., 2002; He et al., 2005]. As the TFF1 promoter has one Sp1 binding site, we expected that either Sp1 or Sp3 but not both would be associated with the TFF1 promoter. To test this idea we used a re-ChIP assay with antibodies against Sp1 and Sp3. First, the ChIP assay was performed using anti-Sp1 or anti-Sp3 antibody. Then, before reversal of protein–DNA cross-linking, the anti-Sp1 ChIP DNA was subjected to re-ChIP using the anti-Sp3 antibody or vice versa. During subsequent PCR only those TFF1 DNA fragments that were simultaneously bound to Sp1 and Sp3 proteins should be amplified. As a control in the re-ChIP assay, the re-ChIP was done with rabbit preimmune IgG. Figure 3A shows that Sp1 and Sp3 do not associate with the same TFF1 promoter in MCF-7 breast cancer cells in the presence or absence of E2. These results demonstrate a TFF1 promoter is bound to either Sp1 or Sp3.

Next we determined whether TFF1 promoters bound to Sp1 or Sp3 were associated with ERα following the addition of E2 using the re-ChIP assay. First, the ChIP assay was performed using anti-ERα antibody. Then, before reversal of protein–DNA cross-linking, the anti-ERα ChIP DNA was subjected to re-ChIP using anti-Sp1 or anti-Sp3 antibodies. As a control, we did not add a second antibody, only beads. During subsequent PCR only those TFF1 DNA fragments that are simultaneously bound to both ERα and Sp1 or Sp3 proteins

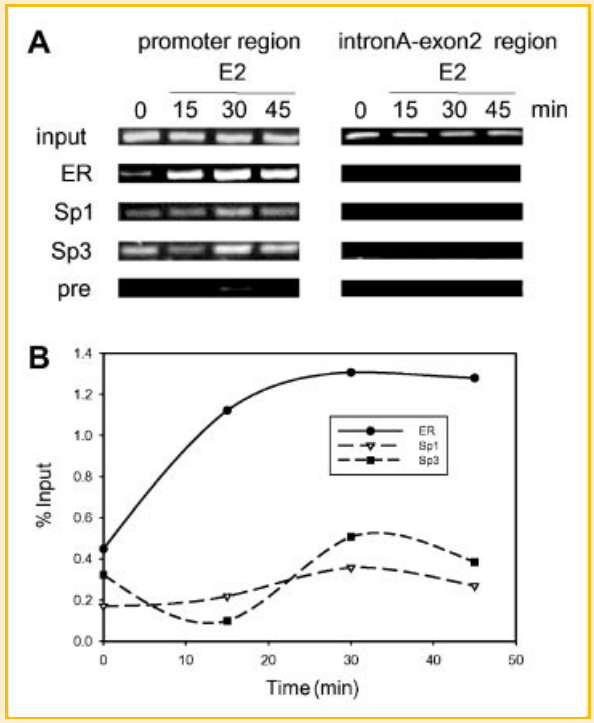

Fig. 2. Temporal loading of ERα, Sp1, and Sp3 following addition of estradiol to MCF-7 cells. A: After 2 h treatment with 2.5 μM α-amanitin, cells were washed and placed in media supplemented with 2.5% dextran charcoal treated FCS including 10 nM E2. Cells were cross-linked with formaldehyde at 15 min intervals up to 45 min. ChIP assays were performed with anti-ERα, anti-Sp1, anti-Sp3 and rabbit preimmune (negative control) antibodies. Input and ChIP DNA were analyzed by primers to the TFF1 promoter and to the intron A–exon 2 (see Fig. 1A). B: The amount of ChIP TFF1 sequence was quantified by real-time PCR as indicated in Materials and Methods Section and presented as % of input. The result is representative of an experiment repeated with three separate preparations.

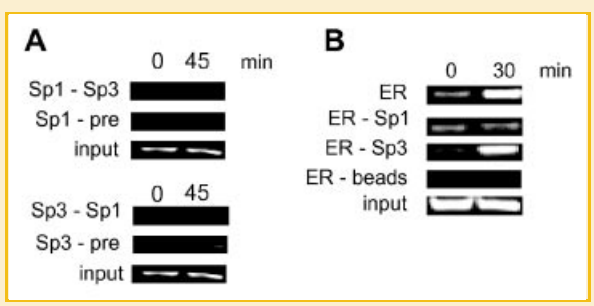

Fig. 3. Co-occupancy of ER with Sp1 or Sp3 on the TFF1 promoter. MCF-7 cells grown in E2-deprived medium were first treated with 2.5 μM α-amanitin for 2 h and then incubated with E2 for 30 or 45 min and were further subjected to re-ChIP assay. A: For the first round of ChIP, the formaldehyde cross-linked chromatin was first ChIPed with anti-Sp1 or anti-Sp3 antibodies followed by a second round of ChIP with anti-Sp3 or anti-Sp1 antibodies, respectively. As a control, the second round of ChIP assays was also done with rabbit preimmune antibodies (pre). Input and re-ChIP DNA were analyzed by primers to the TFF1 promoter B: For the first round of ChIP, the formaldehyde cross-linked chromatin was first ChIPed with anti-ERα antibodies followed by a second round of ChIP with anti-Sp1, anti-Sp3 antibodies or no antibodies (negative control). Input and ChIP DNA were analyzed by primers to the TFF1 promoter.

should be amplified. Figure 3B shows that the co-occupancy of Sp1 and ERα on TFF1 promoter remains at a similar level regardless of the presence of E2. However, much higher extent of Sp3 and ERα co-occupancy on the TFF1 promoter was observed in the presence of E2. These results suggest that the TFF1 promoter bound to ERα is associated with Sp1 or Sp3, with ERα bound TFF1 promoters tending to favor co-occupancy with Sp3.

Sp3 AND SERINE 5 PHOSPHORYLATED RNA POLYMERASE II CO-OCCUPY TFF1 PROMOTER AFTER E2

As Sp3 may function as an activator or a repressor, we determined whether a Sp3 bound TFF1 promoter would support transcription. To address this question we applied the re-ChIP assay to find out if a TFF1 promoter bound to Sp3 was also associated with the serine 5 phosphorylated form of RNA polymerase II, the initiating form of the enzyme. The assay was performed with anti-Sp1 or anti-Sp3 antibody for the first ChIP and anti-phosphorylated serine 5 of RNA polymerase II for the re-ChIP. As a control, rabbit preimmune IgG was used in the re-ChIP. Figure 4 shows that a TFF1 promoter bound to Sp1 was associated with serine 5 phosphorylated RNA polymerase II in cells treated with E2. Similarly a promoter associated with Sp3 also had serine 5 phosphorylated RNA polymerase II present. This result suggests that a TFF1 promoter associated with Sp1 or Sp3 is transcriptionally active, and provides evidence that Sp3 is acting as an activator in the E2-induced expression of the TTF1 promoter.

DISCUSSION

The TFF1 promoter has a Sp binding site positioned next to a ERE. Others and we have demonstrated that Sp1 and Sp3 are associated with the TFF1 promoter in E2 responsive breast cancer cells before and after E2 addition [Sun et al., 2005; Higgins et al., 2006; Khan et al., 2007]. The association of Sp1 and Sp3 with the promoters of E2 responsive genes in breast cancer cells appears to be a common feature, regardless of whether the promoter has an ERE or not [Higgins et al., 2006; Khan et al., 2007]. However, in re-ChIP assays, we show that it is either Sp1 or Sp3 but not both binding to the TFF1

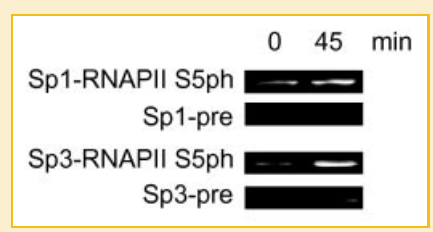

Fig. 4. Co-occupancy of serine 5 phosphorylated RNA polymerase II and Sp1 or Sp3 in the TFF1 promoter. MCF-7 cells grown in E2-deprived medium were first treated with 2.5 μM α-amanitin for 2 h and then were treated with E2 for 45 min. The formaldehyde cross-linked chromatin was first ChIPed with antibodies against Sp1 or Sp3. The ChIPed DNA isolated with the Sp1 or the Sp3 antibody were submitted to a second round of ChIP with antibodies against serine 5 phosphorylated RNA polymerase II (RNAP II S5ph) or rabbit preimmune (pre) antibodies. Input and ChIP DNA were analyzed by primers to the TFF1 promoter.

promoter in MCF-7 cells. As Sp1 and Sp3 have different nuclear locations in MCF-7 cells, it is possible that a TFF1 promoter occupancy with Sp1 or Sp3 may be dependent upon the promoter's nuclear position next to a Sp1 or Sp3 foci.

Our study provides evidence that Sp3 preferentially co-occupied the TTF1 promoter with ERα. Further we demonstrated that the Sp3 occupied promoter supported transcription demonstrating that in the context of this E2 regulated promoter, Sp3 was acting as an activator rather than a repressor. Consistent with our results, several studies have shown that Sp3 has a prominent role in E2-induced expression of genes in breast cancer cells. Higgins et al. found that in E2-responsive ZR-75 cells that Sp3 and Sp1 was bound to the E2-responsive vascular endothelial growth factor receptor-2 promoter (VEGFR2) before and after the cells were treated with E2, and through knock down studies found that ERα/Sp3 played a critical role, while ERα/Sp1 had a minimal role in transactivation of the VEGFR2 promoter. In contrast to the TFF1 promoter, the VEGFR2 promoter lacks an ERE and has multiple Sp binding sites [Higgins et al., 2006]. Sp3 was also found to be required in the E2-induced expression of the retinoic acid receptor α and carbamoylphosphate synthetase/aspartate transcarbamylase/dihydroorotase promoters [Khan et al., 2007].

Others and we have demonstrated that Sp1 or Sp3 associate with ERα and that this interaction was ligand independent [He et al., 2005; Higgins et al., 2006; Khan et al., 2007]. However, our observations on the localization of ERα, Sp1 and Sp3 in the interphase MCF-7 nucleus show that a minor population of ERα binds to Sp1 or Sp3 [He et al., 2005]. Our re-ChIP assays provide evidence that ligand bound ERα tended to be associated with Sp3 rather than Sp1 on the TFF1 promoter. There are several properties of Sp1 that distinguish this transcription factor from Sp3 that may explain our observation. In addition to having different locations in the nucleus and nuclear matrix, Sp1 but not Sp3 can form multimers [Li et al., 2004; He et al., 2005]. As the Sp binding site is positioned close to the ERE in the TFF1 promoter, a Sp1 multimeric complex may sterically interfere with the binding of ERα, providing a possible explanation for the preferred co-occupancy of E2-bound ERα and Sp3 on the promoter. There are several E2 responsive promoters that have multiple Sp binding sites but are lacking an ERE. In this promoter context, ERα is acting as a coactivator in that it is not binding to the promoter DNA directly but is being recruited to the promoter by Sp1 and/or Sp3 [Castro-Rivera et al., 2001; Higgins et al., 2006; Khan et al., 2007]. In such promoter contexts, the Sp1 multimer may associate with one or more ERα factors, and this arrangement may be preferred over a Sp3 association with a single ERα. Re-ChIP studies will be required to sort out the preferred associations of Sp1 family members with E2 bound ERα in different promoter contexts found in E2 responsive genes in breast cancer cells.

ACKNOWLEDGMENTS

This work was supported by CancerCare Manitoba Foundation, Inc., National Cancer Institute of Canada (funds from the Canadian Cancer Society), and Canada Research Chair (to JRD).

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