Binding of estrogen receptor α/β heterodimers to chromatin in MCF-7 cells

Zoi Papoutsi, Chunyan Zhao, Milica Putnik, Jan-Åke Gustafsson and Karin Dahlman-Wright

Department of Biosciences and Nutrition, Karolinska Institutet, NOVUM, SE 14157 Huddinge, Sweden

(Correspondence should be addressed to Z Papoutsi; Email: zoi.papoutsi@ki.se)

Abstract

Estrogen receptors (ERs), ER α and ER β , belong to a group of transcription factors that, upon ligand binding, regulate gene expression by binding to specific DNA regions in chromatin as dimers. In this article, we applied the sequential chromatin immunoprecipitation assay (Re-ChIP) to study the simultaneous presence of ER α and ER β on various DNA-binding regions in intact chromatin. ER α/β heterodimers were isolated by precipitation with anti-ER β antibody followed by anti-ER α antibody from a stable MCF-7-derived cell line that expresses endogenous ER α and an inducible version of ER β . The Re-ChIP method was first validated based on the detection of ER α/β heterodimers bound to a promoter region of the *pS2* gene known to bind both ER α and ER β . We next examined 12 ER-binding sites using Re-ChIP assays for ER α/β heterodimer recruitment. Our results confirmed the recruitment of ER α/β heterodimers to all these regions. This study represents the first demonstration of binding of ER α/β heterodimers to various DNA-binding regions in intact chromatin.

Journal of Molecular Endocrinology (2009) **43**, 65–72

Introduction

Estrogen receptors (ERs), ER α and ER β , mediators of the biological actions of estrogens, belong to the nuclear receptor protein family. In the classical model of estrogen action, upon ligand binding, the receptor dimerizes, and binds to DNA, followed by recruitment of co-regulatory proteins and transcriptional regulation of target genes. ER α and ER β are highly homologous, displaying 96 and 58% homology in their DNA- and ligand-binding domains (LBD) respectively. The region that is responsible for receptor dimerization lies within the LBD (Dahlman-Wright *et al.* 2006). Although ER α and ER β are widely expressed throughout the body, they exhibit distinct expression patterns in a variety of tissues. ER α is expressed primarily in the uterus, kidney, and liver, whereas ER β is highly expressed in the prostate, lung, bladder, and central nervous system (Matthews *et al.* 2006).

The fact that there are two ERs suggests that heterodimerization (α/β) could play a role in estrogen signaling. The formation of ER α/β heterodimers has been demonstrated using techniques such as glutathione-S-transferase (GST) pull-down assays (Pace *et al.* 1997, Pettersson *et al.* 1997, Ogawa *et al.* 1998) and gel shift assays (Cowley *et al.* 1997, Tremblay *et al.* 1999). The chromatin immunoprecipitation (ChIP) assay is a powerful tool to study protein:DNA interactions in their native chromatin context (Collas & Dahl 2008). Several recent studies using ChIP assays have explored

the binding of ER α and ER β to DNA in intact chromatin in MCF-7 cells (Carroll *et al.* 2006, Liu *et al.* 2008). However, the association of ER α/β heterodimers to DNA was not investigated in these studies. Using sequential ChIP with two antibodies (Re-ChIP), the simultaneous binding of two proteins to the same DNA-binding region can be demonstrated in the context of intact chromatin (Kouskouti & Talianidis 2005).

In this article, we describe our studies of the binding of ER α/β heterodimers to various DNA-binding regions in intact chromatin, applying the Re-ChIP method to a stable MCF7-derived cell line that expresses endogenous ER α and an inducible version of ER β .

Materials and methods

Generation of tetracycline-inducible stable cell lines

MCF-7 Tet-Off cells were purchased from Clontech and maintained in DMEM/F-12 supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 μ g/ml G418 (Invitrogen). The cells were transfected with either the parental plasmid (pBI-EGFP) to generate a mock cell line or a plasmid (pBI-EGFP-ER β) expressing a flag-tagged human ER β using the Lipofectamine 2000 reagent (Invitrogen). Cells were selected in the presence of 5 μ g/ml blasticidin for 10 days. ER β protein levels were determined by western blot analysis. In order to confirm the functionality of the ER β protein, we examined its effect on cell proliferation.

We evaluated the cell cycle distribution by fluorescence activated cell sorter analysis (FACS) in both MCF-7 mock and MCF-7 ERβ expressing cells after 72-h stimulation with E₂. The FACS analysis revealed a decrease in the percentage of cells in the S phase in MCF-7 ERβ cells, 12% compared with 18% in MCF-7 mock cells (data not shown). This finding is in line with previously published data, where overexpression of ERβ inhibited the proliferation of breast cancer cells (Chang *et al.* 2006, Williams *et al.* 2008).

RNA purification and quantification of mRNA levels

To assay ERα and ERβ mRNA levels, MCF-7 ERβ and MCF-7 mock cells were seeded in six-well plates and grown in the absence of tetracycline (–tet, +ERβ) for 4 days. To assay mRNA levels of ER target genes, MCF-7 ERβ and MCF-7 mock cells were seeded in six-well plates and grown in 5% FBS dextran-coated charcoal-treated DMEM phenol red free media in the absence of tetracycline (–tet, +ERβ) for 2 days, followed by incubation with 10 nM E₂ for 24 h. Total RNA were purified using the RNeasy Mini Kit (Qiagen). Two microgram total RNA from each well was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) with random hexamer primers. Real-time (RT) PCR assays were conducted using the Applied Biosystems 7500 fast RT PCR system with SYBR green master mix RT-PCR reagent (Applied Biosystems, Stockholm, Sweden). All RT-PCRs were performed in duplicate. Acidic ribosomal phosphoprotein PO (36B4) was used as internal control gene (Akamine *et al.* 2007). The sequences of the primers are listed in Table 1.

Western blot analysis of ERα and ERβ protein levels

MCF-7 ERβ and MCF-7 mock cells were seeded in 150 mm dishes and grown in the absence of tetracycline (–tet, +ERβ) for 4 days. Total cell extracts were

prepared as previously described (Zhao *et al.* 2007). Aliquots corresponding to 40 µg cell extract and 1–10 ng human recombinant ERα and ERβ (Invitrogen) were separated by SDS/PAGE. For quantification of ERβ and ERα protein levels, we used the anti-ERβ rabbit polyclonal antibody LBD, developed in our laboratory (Omoto *et al.* 2001) and the anti-ERα rabbit polyclonal antibody HC-20 (Santa Cruz, Stockholm, Sweden). Protein bands were quantified with densitometry using Scion Image Software, Maryland, USA.

Chromatin immunoprecipitation/re-immunoprecipitation

MCF-7 ERβ and MCF-7 mock cells were seeded in 150 mm dishes and grown in the absence of tetracycline (–tet, +ERβ) for 4 days. Cells were treated with 10 nM E₂ for 45 min and ChIP was performed as previously described (Matthews *et al.* 2006, Liu *et al.* 2008). The anti-ERβ rabbit polyclonal antibody LBD (Omoto *et al.* 2001) was used to perform ChIP for ERβ and the rabbit polyclonal anti-ERα antibody HC-20 (Santa Cruz) was used for ERα ChIP. The rabbit polyclonal anti-RNA polymerase II antibody H-224 (Santa Cruz) was used for RNA polymerase II ChIP. Normal rabbit IgG was used for the determination of non-specific binding. For Re-ChIP, ChIP was performed with the anti-ERβ LBD antibody or the anti-ERα antibody HC-20. Beads from the first cycle of ChIP were incubated with an equal volume of 10 nM dithiothreitol at 37 °C for 30 min, centrifuged at 16 000 g for 1 min to elute DNA-bound proteins. The elution was repeated twice. The final elute was diluted 1:15 in lysis buffer containing a protease inhibitor cocktail (Roche) and re-immunoprecipitated with the anti-ERα HC-20 antibody, the anti-Pol II H-224 antibody, or normal rabbit IgG. The final pellet was processed for western blotting (ChIP protein) or, after reverse cross-linking, for PCR or RT PCR (ChIP DNA).

Table 1 Primer pairs employed to quantify mRNA levels of estrogen receptors (ERα, ERβ), the internal control gene, acidic ribosomal phosphoprotein PO (36B4), as well as of some selected genes

	Forward	Reverse
ERα	GAA TCT GCC AAG GAG ACT CGC	ACT GGT TGG TGG CTG GAC AC
ERβ	TGT CTG CAG CGA TTA CGC A	GCG CCG GTT TTT ATC GAT T
36B4	GTG TTC GAC AAT GGC AGC AT	GAC ACC CTC CAG GAA GCG A
pS2	CATCGACGTCCCTCCAGAAGAG	CTCTGGGACTAATCACCGTGCTG
NBPF1	CACAGCACCTTGTC AAAAGC	TGAACATCTTCATCGTCATCGTT
NBPF4	TGTTGCCAGGGATTACTCCAA	AGATCACTTTGCTTTTCGAGGAA
NOTCH2	GGAACAGCAAGACATGCAA	TGGTCACAGTGGTTGTCTTTGAA
BCL9	CCAAACGATGACTCTGACATTAAAGA	TGGTGTGTGCTGGGAATCC
PRUNE	CAGAGATCTTGGACAGGCAAAC	TGTTGACACAGTCCAGGATGATG
ADORA1	TTCCACACCTGCCTCATGGT	GCGGTCCACAGCAATTGC

Re-ChIP followed by western blotting, PCR or RT PCR

Total ChIP protein was eluted from beads and separated by SDS/PAGE followed by detection with the anti-flag M5 monoclonal antibody (Sigma). Equal amounts of PCR product after amplification with pS2 primers (Table 2) were analyzed by gel electrophoresis and visualized by ethidium bromide staining. Immunoprecipitated DNA was amplified by RT PCR using Platinum SYBR green quantitative PCR supermix uracil DNA glycosylase (Invitrogen). The primer sequences for the 14 genes that were investigated are given in Table 2.

Results

The MCF-7 ER β cell line expresses similar levels of ER α and ER β

A stable cell line, MCF-7 ER β , that expresses an inducible version of ER β fused to a flag tag, was used in all experiments. The MCF-7 mock cell line, a clone of MCF-7 stably transfected with the empty vector, was used as a negative control for ER β expression. The cell lines express endogenous ER α . Initially, we measured the mRNA levels of both ER α and ER β at conditions similar to those of the ChIP and Re-ChIP experiments. As shown in Fig. 1A, the MCF-7 mock cell line and the MCF-7 ER β cell line express equal amounts of ER α mRNA levels. In the MCF-7 ER β cell line, ER β mRNA levels were similar to those of ER α , while the ER β mRNA levels were undetectable in the MCF-7 mock cell line. Protein levels of ER α and ER β were quantified, under the same conditions, by western blot analysis using purified ER α and ER β proteins as standards. As shown in Fig. 1B, high levels of ER β protein were observed in the MCF-7 ER β cell line, whereas no expression of ER β was detected in the MCF-7 mock cell line. In Fig. 1C, the

amounts of expressed ER α and ER β proteins are shown as derived from comparison with those of the corresponding purified proteins of known concentration. This analysis revealed that the protein ratio of ER α :ER β is 07:1 in the MCF-7 ER β cell line under the conditions used in our ChIP and Re-ChIP experiments.

Binding of ER α / β heterodimers to DNA in intact chromatin

To demonstrate binding of ER α / β heterodimers to intact chromatin, we performed Re-ChIP experiments. ER α / β heterodimers were isolated by precipitation with the anti-ER β antibody followed by the anti-ER α antibody. DNA-bound ER α / β was confirmed by western blot analysis showing that ER β can be detected in protein-DNA complexes after sequential precipitation with anti-ER β and anti-ER α antibodies. The interaction of ER α / β heterodimers with specific DNA-binding regions was further assessed by conventional PCR targeting a region of the pS2 promoter known to bind ER α and ER β (Fig. 2A). Additionally, binding to the pS2 promoter region, after sequential immunoprecipitation with ER β and ER α antibodies, was confirmed using RT PCR. As shown in Fig. 2B, a significant recruitment of ER α to the pS2 promoter was observed in the MCF-7 mock cell line, while ER α , and ER β , as well as ER α / β heterodimers, were recruited to the pS2 promoter in the MCF-7 ER β cell line.

Our group has recently identified genome-wide ER α - and ER β -binding regions in the MCF-7 ER β cell line by the ChIP-on-chip assay (unpublished data). Based on this information, 12 regions (each labeled by the closest gene in Fig. 3) that were identified as binding regions for both ER α and ER β with similar signal intensity in the ChIP-on-chip analysis (ratio of ChIP sample to input sample) were selected for further studies of the

Table 2 Primer pairs for amplification of chromatin immunoprecipitation assay (ChIP) and Re-ChIP enriched regions

	Forward	Reverse
18S	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC
pS2	CCT CCC GCC AGG GTA AAT AC	CCG GCC ATC TCT CAC TAT GAA
NBPF1	AGC GGC ATC CCC AGT GT	AGG GTG CCT GGA GAT TAA GC
NBPF4	GGC TGC AGA GGT GGG TAT GA	GCC CCA TTG GCA GAT CAC T
VANGL1	GGC TTG GCT TGT GCT ACG A	AGT TAG GGT GGC AGC AAC CTT
NOTCH2	CCA TTC TTG CTC TTG CCT TAG AG	GCT TGT GGC CAT GTT TTG C
NOTCH2NL	CAG GGC ATC AGA TTC CAT CGA	TGA GAG CCT GTT TTC TTG AGC AT
BCL9	TTG ACA GAA GCC CTG GAG ATG	GCC TGC CAG GTT TCA GGA A
NBPF15	CTT GTT TTA GCT CAT CTG TCC ATC A	GGG TGA AAA GTC AGC CGT TTA T
PRUNE	GAG AGC AAA AAG CCA AGG TTA CA	ACA GCC TCG GCA AAT ATC TTC T
PBX1	TTC CTG CCG GCT TTG TGG	GCC TGC CGT GAC CTC TTC TA
NR5A2	AAG CAA AGA GAT GAT GGA TGT AAC TC	CCT TTT TCC TCC CCA GTT GTG
ADORA1	GAT GGA TGG GAA CAC ATT GGT	TGG TGG CGG AGC ACA AA
CAPN2	CTG GCA TCT GCC TAC AGC AA	GTG TCA TTG TGC CCC AAC AG

Figure 1 (A) ER α and ER β mRNA levels in MCF-7 mock and MCF-7 ER β cell lines. Cells were grown in the absence of tetracycline for 4 days. Data are presented as relative mRNA levels compared with acidic ribosomal phosphoprotein PO (36B4). Results represent the mean $\pm$ s.d. values of two independent experiments conducted in six replicates. (B) ER α and ER β protein levels in total cell extracts from MCF-7 ER β cell lines determined by western blot analysis. Cell extracts were separated by SDS/PAGE. Anti-ER β and anti-ER α antibodies were used for detection as indicated. (C) Quantification of ER α and ER β protein levels in MCF-7 cells were based on standard curves (1–10 ng protein) made with pure recombinant ER α and ER β proteins. Purified and expressed protein bands were quantified by densitometry using Scion Image Software.

binding of ER α / β heterodimers to DNA. Regions from the pS2 promoter and the *18S* gene, which serve as ER-bound positive control and ER-bound negative control respectively were also included in the experiments.

The binding of ER α and ER β to these DNA-binding regions was confirmed using ChIP followed by RT PCR (Fig. 3). All the binding sites exhibited similar fold enrichment for both ERs. There was no recruitment of any ER to a region of the *18S* gene in the MCF-7 ER β cell line. In the MCF-7 mock cell line, with no expression of ER β , there was no detectable recruitment of ER β to any of the binding sites tested, while the

recruitment of ER α was similar to that in the MCF-7 ER β cell line (data not shown).

To study the binding of ER α / β heterodimers to these regions in the context of intact chromatin, we performed Re-ChIP experiments. Re-ChIP was performed with the anti-ER β antibody followed by the anti-ER α antibody and precipitated DNA was analyzed using RT PCR. In the MCF-7 ER β cell line, all analyzed binding regions from the pS2 (trefoil factor 1), neuroblastoma breakpoint family, member 4 (*NBPF4*), NOTCH *Drosophila* homolog 2 (*NOTCH2*), NOTCH *Drosophila* homolog 2 N-terminal like (*NOTCH2NL*), *NBPF15*, *PRUNE* (*Drosophila* h-prune homolog), B-cell chronic lymphoblastic leukemia/lymphoma 9 (*BCL9*), pre-B-cell leukemia homeobox 1 (*PBX1*), nuclear receptor subfamily 5, group A, member 2 (*NR5A2*), adenosine A1 receptor (*ADORA1*), calpain 2 (*CAPN2*), *NBPF1*, and Van Gogh-like 1 (*VANGLI1*) genes exhibited significant fold enrichment of ER α /ER β heterodimers, whereas there was no enrichment to the analyzed region of *18S* (Fig. 4).

Figure 2 (A) Experimental design for the Re-ChIP assay. Confirmation of ER α / β heterodimers was based on the flag tag detection of the ER β protein by western blotting and on the amplification of a fragment from the pS2 promoter by conventional PCR. (B) ER α , ER β , and ER α /ER β recruitment to the pS2 promoter. MCF-7 mock and MCF-7 ER β cells were treated and processed for ChIP and Re-ChIP assays as described in Materials and methods. Binding of ER α , ER β , and ER α /ER β in immunoprecipitated fractions to a fragment from the pS2 promoter was confirmed by real-time PCR. Data are presented as fold enrichment of ER α , ER β , and ER α /ER β binding compared with IgG (means $\pm$ s.d., $n=2$).

Figure 3 ER α and ER β recruitment to DNA-binding regions. MCF-7 ER β cells were treated and processed for ChIP assays as described in Materials and methods. Real-time PCR was performed to confirm the binding of ER α and ER β to various DNA-binding regions in immunoprecipitated fractions as indicated. Data are presented as fold enrichment of ER α or ER β binding compared with IgG (means $\pm$ s.d., $n=2$). A fragment from the pS2 promoter was used as positive control while a fragment from the 18s was used as negative control for ER binding.

Recruitment of RNA polymerase II to ER α / β -bound DNA regions

To investigate the effect of ER β on transcriptional regulation and in particular on the recruitment of RNA polymerase II, we performed ChIP experiments using an anti-PolII antibody. As shown in Fig. 5A, PolII is recruited to the pS2 promoter in both the MCF-7 mock and the MCF-7 ER β cell lines. However, the PolII recruitment is significantly decreased by ER β expression in the MCF-7 ER β cell line (Fig. 5A). Re-ChIP assays (ER β /PolII, ER α /PolII and ER β /ER α) were used to assay co-recruitment of ERs and RNA polymerase II to the pS2 promoter. As shown in Fig. 5B, a significant co-recruitment of ER α and PolII to the pS2 promoter was observed in the MCF-7 mock cell line, while, as expected, there was no co-recruitment of ER β and PolII or ER α and ER β in this cell line. In the MCF-7 ER β cell line, significant recruitment to the pS2

promoter was observed for all three Re-ChIP assays, suggesting that PolII co-exists with ER α and ER β on this binding site.

Estrogen treatment affects expression of genes close to ER α / β -bound DNA regions

To assay whether the presence of ER β affects the expression of ER α -mediated transcription, we compared the mRNA levels of seven selected genes in the MCF-7 ER β cell line versus the MCF-7 mock cell line in response to E₂ treatment and increased expression of all tested genes, following E₂ treatment, was observed in the MCF-7 mock cell line (Fig. 6). Interestingly, in the presence of ER β , the expression of pS2, ADORA1, NBP1, and NBP4 was decreased, the expression of PRUNE and NOTCH2 not changed while the expression of BCL9 was increased.

Figure 4 ER α / β heterodimer recruitment to DNA-binding regions. MCF-7 ER β cells were treated and processed for Re-ChIP assay as described in Materials and methods. Real-time PCR on DNA from ER α / β immunoprecipitated fractions was performed on regions from various genes as indicated. Data are presented as fold enrichment of ER α / β binding compared with IgG (means $\pm$ s.d., $n=2$). A fragment from the pS2 promoter was used as positive control while a fragment from the 18s was used as negative control for ER binding.

Figure 5 (A) PolII recruitment to pS2 promoter. (B) ER β /PolII, ER α /PolII, and ER β /ER α recruitment to pS2 promoter. MCF-7 mock and MCF-7 ER β cells were treated and processed for ChIP or Re-ChIP assays as described in Materials and methods. Binding of PolII alone or ER β /PolII, ER α /PolII, and ER β /ER α in immunoprecipitated fractions to a fragment from the pS2 promoter was confirmed by real-time PCR. Data are presented as fold enrichment of PolII alone or ER β /PolII, ER α /PolII, and ER β /ER α compared with IgG (means $\pm$ s.d., $n=2$).

Discussion

In this study, we report the identification of ER α / β heterodimer-binding DNA regions in MCF-7 ER β cells that express an inducible version of ER β and endogenous ER α , using a Re-ChIP assay. As a negative control, we used a mock MCF-7 cell line. Although the

Figure 6 mRNA levels of selected genes in MCF-7 mock and MCF-7 ER β cell lines. Cells were treated and processed for RNA extraction and analysis of mRNA levels as described in Materials and methods. Data are presented of E $_2$ treated cells relative to vehicle treated cells after normalization to acidic ribosomal phosphoprotein PO (36B4). Results represent the mean $\pm$ s.d. values of two independent experiments conducted in six replicates.

formation of ER α / β heterodimers has been previously demonstrated using GST-pull down and gel-shift assays (Pace *et al.* 1997, Pettersson *et al.* 1997), the exact role of ER α / β heterodimers in estrogen signaling remains largely unknown. However, it is assumed that in cell types, where the two receptor subtypes are co-expressed, the formation of ER α / β heterodimers plays an important role in E $_2$ signaling (Matthews *et al.* 2006).

Recent studies have explored the ER α - and ER β -binding sites for whole genomes or for specific chromosomes (Carroll *et al.* 2006, Liu *et al.* 2008); following up on these studies, we studied ER α / β heterodimers in a cell system where both ER α and ER β are expressed. It has been observed previously that the extent of formation of ER α / β heterodimers in a system, with both ERs are present, depends upon the relative amount of contributing receptors (Li *et al.* 2004). Under conditions of co expression of ERs at similar levels, we applied a Re-ChIP assay, followed by RT PCR, to characterize in a quantitative manner the recruitment of ER α / β heterodimers to various DNA-binding regions in intact chromatin. We detected, by western blotting the ER β protein only in the ER β -ER α and not in the ER β -IgG immunoprecipitated samples after Re-ChIP assay (Fig. 2A). We confirmed the presence of ER α / β heterodimers on the pS2 promoter, a well-characterized promoter to which both ER α and ER β bound in the MCF-7 ER β cell line (Zhao *et al.* 2007). As shown in Fig. 2, amplification of the pS2 promoter was only detected in ER β -ER α immunoprecipitated fractions from the MCF-7 ER β cell line. Previously, Monroe *et al.* (2005) showed binding of ER α / β heterodimers to chromatin in a modified osteoblast cell line by amplifying ER-binding region of two genes, *RBBP1* and *DHCR7*.

In order to study the simultaneous presence of ER α / β heterodimers on various binding sites in intact chromatin, we queried ER-binding sites recently mapped by the ChIP-on-chip assay (data not shown) and randomly selected 12 DNA regions, which showed similar signal intensity (ratio of ChIP sample to input sample) in the ChIP-on-chip assay for ER α and ER β . Notably, eight binding sites are located within genes (*NBPFI*, *NBPFI4*, *VANGL1*, *NOTCH2*, *NOTCH2NL*, *BCL9*, *NBPFI5*, and *PBX1*), and the residual four are located in the promoter regions (*NR5A2*, *ADORA1*, *CAPN2*, and *PRUNE*).

To confirm the recruitment of both ER α and ER β to the regions identified by the ChIP on chip experiment, we performed ChIP assays using ER α or ER β antibodies. All the binding sites tested exhibited significant recruitment of ER α and ER β and the level of recruitment was similar for both receptors. Next, we examined the binding of ER α / β heterodimers to the selected DNA regions using a Re-ChIP assay. In the MCF-7 ER β cell line, all the investigated DNA-binding regions

showed significant recruitment of heterodimers. However, it is notable that different binding sites exhibit different fold enrichment of ER α / β heterodimers. This could be attributed to variations in affinity of heterodimers to various ER-binding sites depending upon the sequence of the binding regions. In a preliminary analysis, using the CONSITE program (Sandelin *et al.* 2004) and setting TF-score cut-off at 85%, we found that all of the selected binding sites contained half-ERE motif, 70% contained AP1-motif (pS2, NOTCH2, NOTCH2NL, PRUNE, BCL9, NR5A2, ADORA1, NBPF1, and VANG1), but only 23% contained full-ERE (pS2, NR5A2, and ADORA1). However, the detailed dissection of the mechanism of heterodimers' recruitment needs a larger number of such identified regions and a systematic binding motifs enrichment analysis with strict background control. Furthermore, our data showed that the ratio of enrichment in the ER α / β Re-ChIP assay versus the enrichment in the ER α and ER β ChIP assays varied. For example, for genes such as *NBPF4*, *NOTCH2*, *NBPF15*, and *PRUNE*, this ratio is high, suggesting that heterodimer recruitment may play a more significant role in the transcriptional regulation of these genes. Our results also showed that overall there is less recruitment of heterodimers compared with ER α or ER β alone. This may be due to the recovery being less after two rounds of ChIP (Re-Chip assay) compared with the single ChIP assay. Additionally, previous kinetic studies have revealed that heterodimers exhibit low binding affinity (Jisa & Jungbauer 2003).

Previous work has shown that the presence of ER β can affect ER α 's transcriptional activity (Chang *et al.* 2006). To verify this in our cell system, we evaluated the mRNA levels of some selected genes after E₂ treatment. Our data showed that mRNA levels of genes that are implicated in cell proliferation, like pS2, and Adora1 are down-regulated, when ER β is expressed (Ribieras *et al.* 1998, Mirza *et al.* 2005). The decrease of pS2 mRNA levels in the MCF-7 ER β cells is in accordance with the decrease of PolIII recruitment to the pS2 promoter in the same cell line as compared with MCF-7 mock cells. The mRNA levels of *NBPF1* and *NBPF4*, whose function is not fully explored, are also decreased in the presence of ER β (Vandepoele *et al.* 2005). mRNA levels of *BCL9*, which is participating in the wnt signaling of cell proliferation and development, were up-regulated in the MCF-7 ER β cell line compared with the MCF-7 mock cell line (Willert & Jones 2006). Finally, the mRNA levels of *NOTCH2* and *PRUNE* were unaffected by the presence of ER β . These genes are both implicated in the aggressiveness of breast neoplasm (Marino & Zollo 2007, Wang *et al.* 2008). Considering that all selected genes exhibit significant ER α / β heterodimer recruitment, our data suggest that ER β may have an activating or inhibitory influence on ER α through heterodimerization. Furthermore, using a

Re-ChIP assay and various combinations of ER α , ER β , and PolIII antibodies, we showed that PolIII co-occupied the same DNA-binding regions as ER α and ER β , consistent with a role of ER α / β heterodimers in transcriptional regulation.

In this study, we report the recruitment of ER α / β heterodimers to various ER-binding DNA regions in an intact cell system, following E₂ treatment. Further experimental data are needed to elucidate the detailed mechanism of formation of ER α / β heterodimers, their function, and the influence of ligands.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from the Swedish Cancer Fund (grant number 08-0573) and from KaroBio AB. Jan-Åke Gustafsson is a shareholder and consultant of KaroBio AB.

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Received in final form 21 March 2009

Accepted 16 April 2009

Made available online as an Accepted Preprint 17 April 2009