REVIEW

Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways

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Abstract

17 β -Estradiol binds to the estrogen receptor (ER) to activate gene expression or repression and this involves both genomic (nuclear) and non-genomic (extranuclear) pathways. Genomic pathways include the classical interactions of ligand-bound ER dimers with estrogen-responsive elements in target gene promoters. ER-dependent activation of gene expression also involves DNA-bound ER that subsequently interacts with other DNA-bound transcription factors and direct ER-transcription factor (protein–protein) interactions where ER does not bind promoter DNA. Ligand-induced activation of ER/specificity protein (Sp) and ER/activating protein-1 [(AP-1); consisting of jun/fos] complexes are important pathways for modulating expression of a large number of genes. This review summarizes some of the characteristics of ER/Sp- and ER/AP-1-mediated transactivation, which are dependent on ligand structure, cell context, ER-subtype (ER α and ER β), and Sp protein (SP1, SP3, and SP4) and demonstrates that this non-classical genomic pathway is also functional *in vivo*.

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Ligand-dependent activation of estrogen receptor (ER)/specificity protein (SP)

Introduction

SP1 was the first transcription factor identified in the early 1980s (Dyban & Tjian 1983, 1985, Briggs *et al.* 1986) and is a member of the Sp/Krüppel-like family (KLF) of at least 25 transcription factors (Suske *et al.* 2005). Sp/KLF proteins have highly variable modular structures in their N-terminal domains and are characterized by three C₂H₂-type zinc fingers in their C-terminal domain, which are required for their sequence-specific DNA binding (Philipsen & Suske 1999, Black *et al.* 2001, Bouwman & Philipsen 2002). Sp/KLF family members recognize GC/GT boxes in promoter regions of mammalian and viral genes, and the consensus SP1 binding site 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3' interacts with most Sp/KLF proteins. SP2 is the major exception among Sp proteins and this protein binds weakly to consensus GC-rich sites but binds with higher affinity to a 5'-GGGCGGGAC-3' motif (Moorefield *et al.* 2004). Interactions of Sp/KLF transcription factors with GC-rich promoters have primarily been investigated with SP1 and SP3 proteins that are ubiquitously expressed in normal

tissues and highly overexpressed in tumors and cancer cells (Philipsen & Suske 1999, Black *et al.* 2001, Bouwman & Philipsen 2002, Suske *et al.* 2005). Both proteins bind the consensus 'SP1' binding sequence and also a large number of non-consensus sequences with variable affinities in *in vitro* binding studies. However, the importance of specific GC-rich sequences in a gene promoter in mediating transactivation is highly variable and dependent on cell context and the relative expression of nuclear coactivators and other cofactors.

Sp proteins play a critical role in several key biological processes including cellular differentiation, proliferation, survival, and angiogenesis (Philipsen & Suske 1999, Black *et al.* 2001, Bouwman & Philipsen 2002), and SP1, SP3, and SP4 knockout animals are either embryolethal or their offspring exhibit severe abnormalities (Supp *et al.* 1996, Marin *et al.* 1997, Gollner *et al.* 2001*a,b*). By contrast, expression of SP1 and possibly other Sp proteins is relatively low in mature animals and decreases with age (Ammendola *et al.* 1992, Oh *et al.* 2007). Several reports show that SP1 and other SP proteins are overexpressed in tumors and cancer cells (Zannetti *et al.* 2000, Shi *et al.* 2001, Chiefari *et al.* 2002, Wang *et al.* 2003, Hosoi *et al.* 2004, Yao *et al.* 2004, Lou *et al.* 2005, Safe & Abdelrahim 2005, Kanai *et al.* 2006), and SP1 overexpression in gastric

tumors is a negative prognostic factor for disease-free survival. For example, Lou *et al.* (2005) reported that transformation of fibroblasts resulted in an 8- to 18-fold increase in SP1 protein expression, and in xenograft experiments, SP1 expression was required for tumor growth. Not surprisingly, recent studies showed that SP1, SP3, and SP4 were highly overexpressed in various cancer cell lines including both ER-positive and ER-negative breast cancer cells (Safe & Abdelrahim 2005, Mertens-Talcott *et al.* 2007), and several reports demonstrate that SP1 and other Sp proteins play an important role in regulation of 17β-estradiol (E₂)-dependent genes in breast cancer cell lines and in many other cell-types (Safe 2001, Safe & Kim 2004).

Role of Sp proteins in hormonal activation of genes

ER/Sp modulation of genes: DNA-dependent interaction of ER and Sp proteins

Many E₂-responsive gene promoters contain both GC-rich estrogen-responsive element (ERE) and ERE half sites (ERE^{1/2}) that cooperatively interact to activate expression of genes. The uteroglobin promoter contains a non-consensus ERE (−263 to −251) that binds ERα; however, hormonal activation of this gene and related promoter constructs involves interaction with both proximal (−232 to −223; −200 to −199) and more distal (−67 to −60) GC-rich Sp binding sites (Scholz *et al.* 1998). Although DNA–protein binding studies have not identified an ERα/Sp complex, both

SP1 and ERα bind individually to the uteroglobin gene promoter oligonucleotide fragments containing the GC-rich and ERE sites respectively. Analysis of the *Xenopus* vitellogenin A1 promoter has also identified ERE and GC-rich sites separated by 1.8 kb that are required for hormonal activation of this gene (Battistuzzo de Medeiros *et al.* 1997). Subsequent studies have identified ERE and GC-rich sites in the estrogen-related receptor and the mouse *Slo* promoter required for hormonal activation (Liu *et al.* 2003, Kundu *et al.* 2007).

Research in this laboratory initially focused on extensive analysis of the cathepsin D gene promoter that had previously been characterized as an E₂-responsive gene (Cavaillès *et al.* 1993, Augereau *et al.* 1994, Krishnan *et al.* 1994, 1995). We identified an E₂-responsive sequence in the −199 to −165 region of the promoter that did not contain ERE motifs. Subsequent analysis identified both a non-consensus ERE^{1/2} and GC-rich elements separated by 23 oligonucleotides (Fig. 1) that were E₂-responsive in transfection assays. Mutation of either the GC-rich or the ERE^{1/2} resulted in the loss of hormone activation of this construct. These results coupled with the detection of an ERα/SP1–DNA complex in gel mobility shift assays clearly demonstrated a novel genomic mechanism of ER-dependent transactivation that required cooperative ERα/SP1 interactions on the cathepsin D promoter. Subsequent studies in several laboratories confirmed that this DNA-dependent ER/Sp-mediated pathway was important for activation of multiple genes, including transforming growth factor α (*Tgfa*), *c-myc*, heat shock protein 27 (*Hspb1*), telomerase,

Figure 1 A summary of E₂-responsive genes containing functional GC-rich and ERE^{1/2} motifs. There is evidence that formation of this complex may contain other proteins.

progesterone receptor A, metastasis-associated protein 3 (*Mta3*), and *Lpr16* (Dubik & Shiu 1992, Porter *et al.* 1996, Petz & Nardulli 2000, Vyhldal *et al.* 2000, Fujita *et al.* 2004, Zhao *et al.* 2005, Boggess *et al.* 2006). Figure 1 illustrates the variety of the GC-rich and ERE $\frac{1}{2}$ motifs, their location in promoters and the number of nucleotides separating the two elements. These motifs and their location are highly variable, making it difficult to predict E $_2$ -responsiveness from these sequences. Moreover, cell context will also influence whether these motifs will form hormone-responsive protein–DNA complexes.

ER/Sp modulation of genes through interactions of Sp proteins with GC-rich motifs

Identification of ER α /Sp-mediated genes. During analysis of the *Hspb1* gene promoter, it was observed that after mutation of the proximal ERE $\frac{1}{2}$ site, transfection of the resulting GC-rich construct in MCF-7 cells followed by treatment with E $_2$ resulted in the induction of reporter gene activity (Porter *et al.* 1997). Subsequent studies with the Hsp27 promoter or a promoter containing one or more consensus GC-rich motifs demonstrated that E $_2$ activated ER α -dependent gene expression through interactions with GC-rich promoter elements (Porter *et al.* 1997). The E $_2$ -dependent activation of ER α /Sp through interactions with GC-rich elements in E $_2$ -responsive gene promoters is a novel genomic pathway for both inducing and repressing gene expression and exhibits several unique characteristics that include the following:

1. ER α directly interacts with the C-terminal DNA-binding domain of SP1, which is a region within SP1 that interacts with a host of other transcription factors including other steroid hormone receptors (Owen *et al.* 1998, Simmen *et al.* 1999, Husmann *et al.* 2000, Lu *et al.* 2000, Curtin *et al.* 2001). Interaction of ER α with other Sp proteins have not been well characterized; however, ER α interacts with multiple domains of SP3 (Stoner *et al.* 2000). ER β interacts with the C-terminal domain of SP1; however, unlike ER α , ER β interacts with regions outside this domain (Saville *et al.* 2000). Interactions between ER and SP1 were ligand-independent.
2. In gel mobility shift assays, a direct formation of ternary ER α /Sp–DNA complexes was not detected. However, ER α ($\pm$ E $_2$) enhanced formation of the Sp–DNA complex and kinetic studies showed that ER α also increased the stability of the SP1–DNA complex.
3. Results of chromatin immunoprecipitation (ChIP) assays showed that ER α is constitutively associated with E $_2$ -responsive GC-rich promoter elements that also bind SP1, SP3, and SP4. Treatment of breast cancer cells with E $_2$ does not appreciably affect ER α and Sp interactions with E $_2$ -responsive GC-rich promoter sequences; however, changes in co-regulatory proteins may be altered.

After initial characterization of GC-rich promoter sites as targets for E $_2$ -dependent transactivation (Porter *et al.* 1997), studies in this laboratory focused on identifying other genes in breast cancer cells that are activated by ER α /Sp. Genes activated by this genomic pathway include *Hspb1*, cathepsin D, *c-fos*, retinoic acid receptor α 1 (*Rara1*), adenosine deaminase, IGF-binding protein 4, *Bcl2*, *E2f1*, thymidylate synthase, vascular endothelial growth factor (*Vegf*), cyclin D1, creatine kinase B, DNA polymerase α , carbamoylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase (*Cad*), ovine oxytocin receptor, and *Vegfr2* (Duan *et al.* 1998, Sun *et al.* 1998, Dong *et al.* 1999, Qin *et al.* 1999, Wang *et al.* 1999, 2002, Xie *et al.* 1999, 2000, Castro-Rivera *et al.* 2001, Samudio *et al.* 2001, Khan *et al.* 2003, Ngwenya & Safe 2003, Stoner *et al.* 2004, Fleming *et al.* 2006, Higgins *et al.* 2006, 2008). Subsequent studies in many other laboratories have extended the list of E $_2$ -responsive genes regulated by ER α /Sp interactions with GC-rich sites and these include folate receptor α , estrogen-related receptor α , prothymosin α , progesterone receptor, prolactin receptor, peptidylarginine deiminase type IV (PADI4), epidermal growth factor receptor (EGFR), kisspeptin 1 (KiSS1), IGF-1 receptor, HOXA10, trefoil factor 1, vitamin D receptor, low density lipoprotein receptor, fibulin-1, rat Sk3, and receptor for advanced glycation end products (Briggs *et al.* 1986, Byrne *et al.* 2000, Salvatori *et al.* 2000, Tanaka *et al.* 2000, Martini & Katzenellenbogen 2001, Jacobson *et al.* 2003, Kelley *et al.* 2003, Schultz *et al.* 2003, Bardin *et al.* 2005, Sun *et al.* 2005, Dong *et al.* 2006, 2007, Maor *et al.* 2006, Li *et al.* 2007, Martin *et al.* 2007). These genes are E $_2$ -responsive in several different cell lines and promoter analysis showed that E $_2$ not only induces gene expression through GC-rich motifs but also other sites in the promoters that may be activated by genomic and/or non-genomic pathways. For example, the *c-fos*, *Bcl2* and cyclin D1 genes all contain E $_2$ -responsive GC-rich sites; however, induction by E $_2$ also involves non-genomic E $_2$ -dependent activation of MAPK/PI3K (*fos*), and cAMP/PKA (*Bcl2* and cyclin D1; Duan *et al.* 1998, 2001, 2002, Dong *et al.* 1999, Castro-Rivera *et al.* 2001; Fig. 2). It is also possible that activation of non-genomic pathways also activates ER/Sp-dependent genes. A recent paper on the induction of PADI4 by E $_2$ in MCF-7 cells also demonstrates the role of multiple hormone-dependent pathways for transactivation (Dong *et al.* 2007). This gene contains two upstream ERE motifs that bind ER α and contribute to induced gene expression; however, the proximal regions of the gene contains GC-rich, AP-1, and NF-YA sites that are important for PADI4 gene expression (Fig. 3). Deletion analysis and RNA interference studies suggest that although ER α /SP1 (and not ER α /SP3) contributes to E $_2$ -induced gene expression, interactions of ER α and/or SP1 with NF-YA and AP-1 are also involved in

Figure 2 Hormonal activation of cyclin D1, *Bcl2*, and *c-fos* in breast cancer cells. In addition to GC-rich sites activated by ER/Sp, E₂-dependent activation of non-genomic cAMP/PKA (cyclin D1 and *Bcl2*), and MAPK/PI3K(*c-fos*) pathways were also identified.

the induction of PADI4 (Dong *et al.* 2007). Similar results were observed for induction of the human prolactin receptor that involved interactions of ER α /Sp with C-EBP β (Dong *et al.* 2006) (Fig. 3).

Studies in this laboratory show that cell context plays a critical role in the molecular mechanisms of hormone-dependent transactivation. E₂ induced E2F1 gene expression in MCF-7 cells and the minimal E₂-responsive region of the E2F1 promoter contained three consecutive GC-rich sites and two CCAAT binding sites (Wang *et al.* 1999, Ngwenya & Safe 2003). Promoter analysis showed that the GC-rich or CCAAT sites alone were not E₂ responsive in transient transfection studies and that hormone-responsiveness required at least one upstream GC-rich site and both CCAAT elements (Fig. 4). DNA binding studies show that ER α and SP1 enhance NF-YA binding to the CCAAT sites and that hormone-induced transactivation in MCF-7 cells is associated with an ER α /Sp/NF-YA complex. Induction of E2F1 in ZR-75 (ER-positive) breast cancer cells involves the same response elements; however, the GC-rich sites are independently activated by ER α /Sp, and NF-YA is induced by E₂ through non-genomic ER-dependent activation of cAMP/PKA.

One of the most striking cell context-dependent differences in hormonal modulation of gene expression by ER α /Sp is associated with expression of *Vegfr2* in ZR-75 and MCF-7 cells (Higgins *et al.* 2006, 2008). *Vegf* and *Vegfr2* are critical angiogenic genes that contain proximal GC-rich sites required for basal and hormone-dependent expression (Fig. 5). Initial studies showed that E₂ induced *Vegfr2* expression in ZR-75 cells and this involved two proximal GC-rich sites at -58 and -54. By contrast, E₂ decreased expression of *Vegfr2* in MCF-7 cells and promoter analysis showed that the same promoter elements required for E₂-induced transactivation in ZR-75 were required for suppressed expression of *Vegfr2* in MCF-7 cells. The major cell

context-dependent differences were observed in ChIP assays where E₂ induced recruitment of the co-repressors NCoR and SMRT to the *Vegfr2* promoter in MCF-7 but not in ZR-75 cells. In the latter cell line, treatment with E₂ increased recruitment of the coactivator SRC-3 to the GC-rich promoter, whereas this was not observed in MCF-7 cells. These observations are consistent with the cell context-dependent differences in hormonal regulation of *Vegfr2*; however, other factors must also be involved and this is currently being investigated.

Role of ER α and ER β in activation of ER/Sp. Initial studies investigated the role of ER α and ER β in activating a GC-rich construct (pSP1) in breast and other cancer cell lines (Saville *et al.* 2000). The results showed that in these cancer cell lines transfected with pSp, ER α , or ER β , E₂ activated ER α /Sp but not ER β /Sp. Subsequent domain swapping experiment with ER α and ER β demonstrated that the N-terminal AF-1 domain of ER α was important for E₂-dependent transactivation, whereas the corresponding A/B domain of ER β fused to the N-terminal C-F domains of ER β or ER α was inactive. These results are consistent with the lack of AF-1 activity in the A/B domain of ER β . Other studies on activation of ER α /Sp versus ER β /Sp are variable and depend on promoter and cell context. ER α but not ER β is involved in activation of PADI4 in HeLa cells (Dong *et al.* 2007); E₂ activates GC-rich EGFR promoter constructs in HeLa cells transfected with ER α or ER β (Salvatori *et al.* 2003). Both ER α and ER β were involved in hormone-dependent activation of GC-rich progesterone receptor promoter constructs; however, the induction response was dependent on cell context (Schultz *et al.* 2005). Kim *et al.* (2005) further investigated ER α -SP1 interactions using fluorescence resonance energy transfer (FRET). Although ER α and SP1 interactions in *in vitro* pulldown assays are ligand-independent, results of FRET studies show that in live MCF-7 cells, E₂ clearly induces ER α -Sp1 interactions.

Figure 3 Multiple E₂-responsive elements. The PADI4 and prolactin receptor genes contain E₂-responsive GC-rich sites and other functional *cis*-element that contributed to E₂-induced transactivation.

Figure 4 Different mechanisms of hormonal activation of *E2f1* in MCF-7 cells and ZR-75 cells.

ER α activation of genes through SP1, SP3, and SP4. Most studies on ER α /Sp-mediated transactivation have assumed that SP1 plays a major role in this response since this protein is overexpressed in cancer cell lines. However, since SP1, SP3, and SP4 proteins are expressed in breast cancer cells, we investigated the effects of individual Sp protein knockdown by RNA interference on the induction of *Rara1*, *E2f1* and *Cad* gene expression by E_2 in MCF-7 cells (Khan *et al.* 2007). All three genes contain GC-rich promoters; however, the role of individual Sp proteins on hormone responsiveness had not been determined. The results showed that knockdown of SP1, SP3, and SP4 significantly decreased the fold induction of RARA1, E2F1 and CAD by E_2 ; however, loss of induction was greater in cells where SP3 or SP4 was decreased compared with decreased SP1. This demonstrates, at least for these three genes, that all three Sp proteins play role in ER α /Sp-mediated transactivation in MCF-7 cells.

Ligand-dependent activation of ER α /Sp. Initial studies showed that in breast cancer cells transfected with pSP1 and wild-type ER α , both E_2 and the antiestrogens, 4-hydroxytamoxifen (4-OHT) and ICI 182 780 (fulvestrant), induced reporter gene (luciferase or chloramphenicol acetyltransferase) activity (Porter *et al.* 1997, Saville *et al.* 2000, Kim *et al.* 2003). By contrast, E_2 but not 4-OHT or ICI 182 780 induced luciferase activity in breast cancer

cells transfected with GC-rich promoter-reporter constructs from several E_2 -responsive genes, demonstrating that promoter structure also influences the ER agonist/antagonist activities of E_2 and antiestrogens. The structure-dependent activation of ER α /Sp was also investigated in MCF-7 and MDA-MB-231 cells transfected with pSP1₃ and wild-type ER α , ER α Δ ZF1, ER α Δ ZF2, ER α (1–553), and ER α (1–537). These ER α mutants contained deletions of zinc finger 1 (amino acids 185–205), zinc finger 2 (amino acids 218–243), the F domain (amino acids 554–595), and the F domain plus amino acids in helix 12 of the E domain (amino acids 538–595) respectively (Fig. 6; Wu *et al.* 2008). The compounds used in this study included E_2 , diethylstilbestrol (DES), antiestrogens, the phytoestrogen resveratrol, and the xenoestrogens octylphenol (OP), nonylphenol (NP), endosulfan, kepone, 2,3,4,5-tetrachlorobiphenyl-4-ol (HO-PCB-Cl₄), bisphenol-A (BPA), and 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). In MCF-7 cells, all compounds except resveratrol induced luciferase activity in cells transfected with wild-type ER α , whereas in MDA-MB-231 cells, even resveratrol was active. The concentrations of each compound were selected based on their maximal inducing response (with wild-type ER α) that was not accompanied by cytotoxicity. E_2 and the xenoestrogens activated wild-type ER α and ER α Δ ZF1/ER α Δ ZF2 in MCF-7 cells, whereas in MDA-MB-231 cells, NP

Figure 5 Mechanisms of E_2 -dependent downregulation and induction of *Vegfr2* in MCF-7 and ZR-75 cells respectively.

Figure 6 Wild-type and ER variants and domains of ER required for transactivation by E₂ and structurally diverse estrogenic compounds in MCF-7 (—) and MDA-MB-231 (---) breast cancer cells.

and OP did not activate luciferase activity in cells transfected with ERαΔZF2 and had minimal effects in cells transfected with ERαΔZF1 (Fig. 6). Thus, with the exception of NP and OP, the xenoestrogens resembled E₂ and DES but not 4-OHT or ICI 182 780. By contrast, 4-OHT and ICI 182 780 but not E₂ or DES induced transactivation in MCF-7 and MDA-MB-231 cells transfected with ERα (1-537), a mutant form of ERα that has lost part of helix 12 that interacts with coactivators. For this form of ERα, the xenoestrogens all induced luciferase activity and resembled the antiestrogenic drugs 4-OHT and ICI 182 780. These results demonstrate that the xenoestrogens can be both ‘estrogen- and antiestrogen-like’, depending on the expression of ERα variants indicating that these compounds are selective ER modulators (SERMs).

RNA interference assays using small inhibitory RNAs for SP1, SP3, and SP4 also demonstrated structure-dependent differences in activation of ERα/SP1, ERα/SP3, and ERα/SP4. ‘Fold induction by estrogens (E₂ and DES), xenoestrogens, and antiestrogens exhibited three patterns that differentially relied upon ERα/SP1, ERα/SP3, ERα/SP4, or their combinations. For E₂, HPTE, DES, and HO-PCB-Cl₄, ERα/SP1 > ERα/SP4, and ERα/SP3 had minimal to no effect on activation of ERα/Sp by these compounds. The pattern of ERα/Sp activation for BPA, endosulfan, NP, and 4-OHT was ERα/SP1 ≈ ERα/SP4 with minimal contributions by ERα/SP3. By contrast, both ERα/SP1 and ERα/SP3 play roles in activation of pSP1₃ by ICI 182 780 and kepone, but ERα/SP4 tends to cause an inhibitory effect since iSP4 enhances the fold induction by these compounds’ (Khan et al. 2007). These results illustrate the complexity of activation of ERα/Sp that depends on ligand structure, ERα variant, and individual Sp proteins. Moreover, in this study, cells were

transfected with the pSP1₃ construct that contains three tandem consensus GC-rich motifs and this pattern of induction will undoubtedly vary with different GC-rich promoter constructs.

Ligand-dependent activation of ER/activating protein-1 (AP-1)

Introduction

AP-1 is a transcription factor complex containing the protooncogenes *jun*, *fos*, and other family members, and this complex interacts with AP-1 sites in gene promoters to activate genes involved in cell growth, differentiation, and development. Early studies showed that *fos* and *jun* suppress ER-dependent transactivation from an ERE promoter (Doucas et al. 1991, Shemshedini et al. 1991, Tzukerman et al. 1991), and there are also reports that E₂ induces expression of AP-1 transcription factors (Duan et al. 1998, 2001, 2002). AP-1 activity is also increased during the progression of MCF-7 cells to an antiestrogen-resistant phenotype, suggesting a role for this complex in E₂-independent and more aggressive breast cancer cells (Dumont et al. 1996). In addition, there was also evidence that hormonal activation of insulin-like growth and ovalbumin involved AP-1 sites and that AP-1 may be important for E₂-dependent activation or repression of the progesterone receptor, gonadotropin-releasing hormone receptor, matrix metalloproteinase-1 (MMP-1), prolactin, and pS2 genes (Van der Burg et al. 1990, Gaub et al. 1990, Phillips et al. 1993, Savouret et al. 1994, Umayahara et al. 1994, Barkhem et al. 2002, Petz et al. 2002, Cheng et al. 2003, Duan et al. 2008, Scafonas et al. 2008). DeNardo et al. (2005) identified 20 new E₂-induced genes that were

AP-1 dependent, and microarrays also identified a subset of 32 ERE-independent differentially expressed genes in breast tumors (Glidewell-Kenney *et al.* 2005). These data confirm the importance of the non-classical genomic pathways for induction of genes by E₂ and other estrogenic compounds. *c-fos* is induced by E₂ through non-genomic and genomic pathways and the former pathway may also contribute to activation of ER/AP-1.

Role of AP-1 proteins in hormonal modulation of gene expression

Estrogen/antiestrogen activation of ER/AP-1

Webb *et al.* (1995) first reported the activation of an AP-1 promoter-reporter construct derived from the human collagenase promoter. Antiestrogens such as ICI 182 780 and 4-OHT and E₂ activated the AP-1 construct in cancer cells (HeLa, NIH-3T3, HepG2, SHM, SY5Y, CEF, CV1, CHO, and F9) derived from several different tissues. In Ishikawa endometrial cancer cells, E₂ and the antiestrogens activated AP-1; however, E₂ but not 4-OHT or ICI 182 780 activated the AP-1 promoter in ER-positive MCF-7 and ZR-75 cells. These results were interpreted as a mechanism by which antiestrogens such as tamoxifen can exhibit ER agonist activity through the non-classical ER/AP-1 pathway in which ER binds *jun* but not *fos* in pulldown assays where the N-terminal AF-1 region of ER α is the major *jun*-interacting site.

Subsequent studies compared the activation of ER α /AP-1 versus ER β /AP-1 by E₂ and selected SERMs that are used for breast cancer or other hormonal therapies (Paech *et al.* 1997). In HeLa cells, the antiestrogens raloxifene, 4-OHT and ICI 182 780 but not E₂ or DES activated ER β /AP-1, and similar results were observed in Ishikawa, MCF-7, and ER-negative MDA-MB-453 breast cancer cells. By contrast, both estrogens and antiestrogens activated ER α /AP-1 in HeLa cells, demonstrating that cell context, ER subtype, and ligand structure were important for activation of ER/AP-1. Interestingly, these results clearly distinguish between ER/AP-1 versus ER/SP where ER β /Sp is relatively inactive and wild-type ER α /Sp is activated by both estrogens and antiestrogens (Fig. 6).

Effects of ER deletion mutants on activation of ER/AP-1

Several studies have investigated the effects of wild-type and variant ER on activation of ER/AP-1 in different cell lines (Webb *et al.* 1995, 1999, Kushner *et al.* 2000, Jakacka *et al.* 2001, Weatherman & Scanlan 2001, Bjornstrom & Sjoberg 2002). It was initially reported that E₂ but not antiestrogens activated HE11/AP-1 where HE11 is a DNA binding domain deletion mutant

of ER α (Webb *et al.* 1995), and this was similar to results obtained for activation of HE11/Sp or the zinc finger deletion mutants ER α Δ ZF1 and ER α Δ ZF2 (Fig. 6). The effects of specific zinc finger mutants within the DNA-binding domain of ER α have also been investigated (Jakacka *et al.* 2001, Bjornstrom & Sjoberg 2002). The ER α E207G/G208S and E207A/G208A mutants do not bind DNA and, in ER-negative TSA cells transfected with these ER α mutants and an AP-1 reporter construct, ICI 182 780 induced luciferase activity and E₂ repressed activity (Jakacka *et al.* 2001). A more extensive study on a series of DNA-binding domain mutants of ER β further demonstrated the complexity of ER α /AP-1- and ER β /AP-1-mediated transactivation in COS-7 and HE11 cells (Bjornstrom & Sjoberg 2002). In HC11 cells transfected with wild-type ER β and an AP-1 reporter construct, E₂ repressed and the antiestrogens 4-OHT and ICI 182 780 induced activity. However, in the same cell line transfected with ER β containing L206A, Y210A, and Δ 122–266 (the entire DBD) mutant and several double mutants, E₂ induced activity and the antiestrogens were inactive. Thus, subtle changes in the ER β DBD completely reversed the ER agonist/antagonist activities of E₂, 4-OHT, and ICI 182 780.

Elegant studies on ER α and ER β deletion mutants and chimeric ERs with interchangeable domains have shown the importance of the C-terminal and N-terminal AF-2 and AF-1 domains on ligand-dependent activation of ER/AP-1 (Webb *et al.* 1999, Weatherman & Scanlan 2001). E₂ induced transactivation of an AP-1 promoter containing only the LBD of ER α , whereas tamoxifen, raloxifene, and ICI 182 780 were inactive. Subsequent studies on deletions of the AF-1 domain of ER α showed that E₂-induced transactivation was dependent on AF-1. The effects of tamoxifen were AF-1 independent but longer N-terminal deletions (129–178) resulted in loss of activity in HeLa cells. Interestingly, deletion of AF-1 from wild-type ER α enhanced tamoxifen-induced transactivation and the response was similar to that observed in HeLa cells transfected with ER β , which does not contain AF-1-dependent activity. However, deletion of the N-terminal A/B domain of ER β resulted in loss of tamoxifen-dependent activation of an AP-1 promoter in HeLa cells (Weatherman & Scanlan 2001). Figure 7 compares the domain requirements for E₂ and antiestrogens for activation of ER α /Sp and ER α /AP-1. Major differences were observed for E₂ that required the AF-1 domain for activation of ER α /Sp; activation of ER α /AP-1 was also dependent on AF-1; however, loss of activity was observed after deletion of aa 127–178 in the N-terminal A/B domain.

Structure-dependent activation of ER/AP-1

The effects of structurally-diverse pharmacologic SERMs and xenoestrogens on activation of ER/AP-1

Figure 7 Domains of ER and ER required for ligand-dependent activation of ER/AP1 (i.e. deletion results in loss of activity) (104, 107) and comparisons with activation of ER/Sp.

have also been reported (Weatherman *et al.* 2001, Walters *et al.* 2002, Fujimoto *et al.* 2004). Differences in activation of ER α /AP-1 and ER β /AP-1 were observed among several drugs being developed for endocrine therapy; however, EC₅₀ values were generally lower for activation of ER β /AP-1 (Weatherman *et al.* 2001). The effects of several estrogenic compounds including xenoestrogens on activation of ERE-luc and AP-1-luc constructs cotransfected with ER α or ER β were investigated in NIH-3T3 cells (Fujimoto *et al.* 2004). Activation of ER α /AP-1 was observed in cells treated with most of the compounds including E₂, 4-OHT, 17 α -estradiol, estriol, dienestrol, bisphenol A, *t*-methyl-butylphenol, kepone, *p,p'*-biphenol, genistein, *o,p'*-DDD, zearalenol and *p,p'*-DDE (but not *p,p'*-DDT), whereas only 4-OHT activated ER β /AP-1. Thus, xenoestrogens primarily activate ER α /AP-1 and some of the same compounds also activate ER α /Sp (Fig. 6).

In vivo studies

Jameson *et al.* initially developed a transgenic mouse model in which a mutant ER α (E207A/G208A; AA) knock-in mouse was generated to investigate promoter DNA-independent estrogenic activity of ER α . The ER α mutant does not bind promoter DNA and therefore a comparison of the effects of E₂ in wild-type and knock-in mutant ER α mice will provide important insights into the DNA (ERE)-independent effects of ER α that could include interactions with AP-1, Sp, and other transcription factors. The knock-in mice (ER⁺/AA) exhibited a gain of function since the females were infertile due to uterine defects and inoovulation (Jakacka *et al.* 2002). These mice have now been bred on an ER α knockout (ER^{-/-}) background to give ER α ^{-/-}/AA mice that can be used in comparative studies with ER α ^{+/+}, ER α ^{-/-}, and ER α ^{-/-}/AA mice to determine ERE-independent responses (O'Brien *et al.* 2006, Glidewell-Kenney *et al.* 2007, McDevitt *et al.* 2007, Syed *et al.* 2007). In the

ER⁻/AA mouse uterus, E₂ and tamoxifen induced luminal epithelial cell proliferation but not other prototypical estrogen responses such as hyperemia and fluid retention (O'Brien *et al.* 2006). The contributions of ERE-independent versus ERE-dependent pathways were also investigated in the mouse reproductive axis. Estrogen-negative feedback control on luteneizing hormone secretion was primarily ERE-independent, whereas positive feedback and ovulatory cyclicity were ERE-dependent processes (Glidewell-Kenney *et al.* 2007). Development of the male skeleton and sexual behavior in mice were primarily ERE-dependent, whereas secretion of testosterone was ERE-independent (McDevitt *et al.* 2007, Syed *et al.* 2007). These results clearly demonstrate an important role for ER/AP-1, ER/Sp, and other DNA-independent estrogenic pathways in mice. However, it is also possible that some of the ERE-dependent/-independent responses may be due to non-genomic pathways and their relative contributions of genomic versus non-genomic E₂-dependent responses in mice and humans requires further research and development of appropriate animal models.

Summary

Ligand-dependent activation of ER is highly complex and dependent on ligand structure, ER subtype and intracellular location, promoter, and cell context. The non-classical genomic ER/Sp and ER/AP-1 regulate a large number of genes through both distinct and overlapping pathways. Studies on the identification of genome-wide ER binding sites have confirmed the association of this hormone receptor with other nuclear factors and binding motifs including GC-rich and AP-1 sites (Carroll & Brown 2006, Carroll *et al.* 2006, Vega *et al.* 2006, Kininis *et al.* 2007, Lin *et al.* 2007, Gao *et al.* 2008). It has also been reported that many

E₂-responsive genes are regulated by interactions of ER with *cis*-elements that are distal to their corresponding transcription start sites. Thus, the molecular biology of E₂-dependent activation of ER/Sp and ER/AP-1 and their associated genes may also include contributions of distal binding sites that have not yet been characterized. The ligand structure- and ER subtype-dependent patterns of activation of ER/Sp and ER/AP-1 are different (Figs 6 and 7), and this may be due, in part, to the relative expression of cofactors required for ER/Sp and ER/AP-1-mediated transactivation. Although coactivator/cofactor requirements for activation of classical ERE promoters have been exhaustively investigated, only a few reports have determined functional coactivator-ER/Sp and ER-AP-1 interactions. Steroid receptor coactivator 2 (or GRIP1) enhances ER α /AP-mediated transcription, and this response requires LXXLL boxes in the coactivator that facilitates interactions with the AF2 domain of ER α (Webb *et al.* 1999). This is consistent with similar pathways for coactivation of ER α (ERE-dependent) and ER α /AP-1 (Webb *et al.* 1999). Receptor-interacting protein 140 (RIP140) repressed E₂-induced activation of ER α /AP-1 by reversing the effects of SRC2 (Teyssier *et al.* 2003). By contrast, studies with the vitamin D-interacting protein 150 (DRIP150) coactivator demonstrated that DRIP150 coactivation of ER α /Sp was LXXLL-box-independent and required a novel helical region in DRIP150 (Lee & Safe 2007). Cell context-dependent induction and repression of *Vegfr2* in ZR-75 and MCF-7 cells respectively is associated with the same GC-rich promoter sequences but there were differences in recruitment of coactivators (induction) and corepressors, such as NCoR and SMRT (Higgins *et al.* 2006, 2008). Further studies are required on the mechanisms and proteins associated with coactivation and repression of ER/Sp and ER/AP-1, since *in vivo* studies demonstrate that these ERE-independent pathways play an important role in mediating the effects of estrogens, pharmacologic, and other synthetic or naturally occurring SERMs.

Declaration of interest

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

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