Journal of Cellular Biochemistry 96:490–505 (2005)

Steroid Hormone Receptor Signaling in Tumorigenesis

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Abstract Excessive activation of the hormone signaling pathways is implicated in several disorders of the target tissues, with cancer being one of the most serious fallouts. Steroid hormone receptors are key proteins through which steroid hormones convey their signals to the cells. Deregulated activity of the hormone receptors due to their altered activation; stability or sub-cellular localization is heavily implicated in the onset and progress of cancers. The role played by estrogen and its receptors in breast cancer remains the most thoroughly investigated steroid-dependent cancer system till date. Choosing it as an example, we have summarized the molecular mechanisms underlying the action of the estrogen receptors (ERs) in manifesting the effects of the estrogens in the cells. A special emphasis is placed on the molecular mechanism of their functionality, role of the coactivator proteins, and the reasons for the deregulated signaling. The therapeutic approaches resulting from the mechanistic study of the ER action and their efficacies are also discussed. J. Cell. Biochem. 96: 490–505, 2005. © 2005 Wiley-Liss, Inc.

Key words: steroid hormone receptors; estrogen receptors; coregulators; transcription; signaling; nongenomic; PELP1

Steroid hormones are a biologically important class of chemically related hormones secreted into the blood stream by the adrenal cortex and gonads (ovaries and testes). Steroid hormones are synthesized from one common precursor molecule, cholesterol, by the involvement of several enzymatic pathways resulting in a myriad of hormones for different target tissues and organs. The synthesis and release of these hormones is a tightly controlled process. Once released into the blood stream, steroid hormones enter target cells by crossing the cytoplasmic membrane and exert their action by binding to high-affinity receptor proteins, called as steroid hormone receptors (SHRs). SHRs are a class of structurally related intracellular proteins that bind to steroid hormones and convey their signals by affecting downstream gene expression. Steroid hormones control a wide variety of cellular functions necessary for cell homeostasis, proliferation, differentiation, and apoptosis. SHRs are crucial for the normal development and growth of their target organs and tissues. Deregulation of steroid hormone secretion, signaling, and SHR action leads to a various disorders, including cancers. Cancers that depend on steroid hormones include breast, prostate, testicular, ovarian, and endometrial cancer [Henderson, 1982]. The concept of a steroid hormone playing a critical role in carcinogenesis was realized long-time back, wherein surgical removal of the ovaries or testes, which reduced the levels of circulating hormones, greatly benefited women with breast cancers [Lerner and Jordan, 1990] or men with prostrate cancer [Huggins and Hodges, 2002], respectively. This review is intended to provide an overview of the involvement of SHRs in the onset and progress of cancers, the role of select coregulatory proteins and the emerging therapeutic approaches to treat hormone-responsive cancers.

SHRs ARE FUNCTIONALLY AND STRUCTURALLY SIMILAR LIGAND-ACTIVATED TRANSCRIPTION FACTORS

The family of SHRs comprises of structurally related proteins, which, on the basis of sequence

Abbreviations used: E2, 17β-estradiol; ER, estrogen receptor; NR, nuclear receptor; PELP1, proline-, glutamic acid-, and leucine-rich protein-1; SHR, steroid hormone receptor; SERMs, selective estrogen receptor modulators.

Grant sponsor: NIH (for work in Kumar's laboratory to R.K); Grant numbers: CA 98823, CA 109379, CA 80066, CA 90970.

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Received 24 May 2005; Accepted 31 May 2005

DOI 10.1002/jcb.20566

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similarity, are believed to form a coherent group of evolutionarily related proteins derived from a common ancestral gene via gene duplication and exon shuffling [Landet, 1997]. Not surprisingly, the members of this family share common structural features, which include a central DNA binding domain (DBD), which is the most highly conserved domain and is comprised of two zinc finger motifs that are involved in DNA binding. The ligand binding domain (LBD) to which the hormones bind is localized in the C-terminal half of the protein and is also referred to as 'activation function 2' (AF2). An extensive structural characterization of the LBD of several receptors like estrogen receptor (ER), progesterone receptor (PR) [Tenenbaum et al., 1998] and androgen receptor (AR) [Shaffer et al., 2004] has resulted in a clear understanding of the binding of the hormone, the resulting conformational changes in the receptor, and the basis of acute specificity of hormone binding to its receptor. The N-terminal region of the SHRs (referred to as activation function 1, [AF1]) has greater sequence variation. Situated between the DBD and LBD is a variable region called as the hinge region. Most receptors also have a variable stretch of amino acids at the C-terminal end whose function is not very well understood (referred to as F domain). The ligand-bound active receptors form homodimers, and the sequence motifs involved in dimerization are present in the DBD and LBD region of the receptors. The binding of the hormone to its receptor results in its activation, nuclear localization, formation of homo or heterodimers, binding to DNA, and modulation of gene transcription. The protein products of these genes are the mediators of steroid signaling pathways.

The specificity of the gene targets affected by steroid hormones is determined by a simple and elegant mechanism, wherein each receptor selectively binds to specific small nucleotide stretches called as 'hormone response elements' (HREs), present in the regulatory chromatin of the target genes. In general, the HREs are composed of two small stretches of six nucleotides (half-sites) separated by one or more variable nucleotides. The identity and specificity of the response elements for different SHRs have been very well studied and characterized [Umesono and Evans, 1989]. There is an underlying similarity in the basic mechanism with which the SHRs function. The present review will attempt to highlight the role of SHRs in the onset and maintenance of cancer with a focus on the role of ER in breast cancer.

ESTROGEN RECEPTORS

Estrogens are major promoters of cell proliferation in both normal and neoplastic breast epithelium [Pike et al., 1983] and mediate their cellular effects via binding to high-affinity ER. Two major ERs have been identified till date, namely ERα and ERβ. ERα was identified in late 1950s and was purified a few years later [Jensen and Jordan, 2003]. ERα functions as estrogen activated transcription factor and involved in the stimulation of estrogen target genes involved in the regulation of cell cycle progression and growth of breast epithelium. Excessive stimulation of the ER pathway due to increased hormonal secretion or increased levels of the receptor may lead to augmentation of cell proliferation and thus increase the risk of uncontrolled growth stimulation and cancer. Patients with breast cancer have elevated levels of ERα expression in comparison to healthy subjects [McGuire et al., 1975]. Results from immunohistochemical studies of normal and cancerous tissue showed approximately 20–30% of cells in normal mammary gland are ER-positive [Khan et al., 1998; Shoker et al., 1999], but the ratio of ER-positive cells significantly increases in proliferative diseases, implying an increased risk of tumorigenesis under conditions of increased ER expression. Several recent studies of differentially expressed estrogen responsive genes in human breast cancer cells and tumors have helped in identifying the possible estrogen-inducible oncogenes [Inoue et al., 2002; Coser et al., 2003; Cunliffe et al., 2003; Frasor et al., 2003]. One such oligonucleotide micro-array study of 19,000 human genes identified 226 estrogen-upregulated genes out of which 137 of them were induced by estrogen and blocked by antiestrogen ICI [Abba et al., 2005]. A comparison of these genes, identified in cell lines, with those in tumors showed that 44 were differentially expressed in at least one breast cancer study, and such genes might have biological and clinical significance to the pathobiology of breast cancer. In another study aimed to identify differentially expressed genes between ERα-positive and ERα-negative primary breast tumors [Chin-Yo et al., 2004], 520 transcripts were identified to be differentially regulated in ERα positive tumors, 473 out of which were up

regulated and 47 were down-regulated. Of the genes with altered expression in ERα-positive cells, 31% were involved in cell growth and maintenance, 21% in cell communication, and 16% in regulation of transcription, highlighting the importance of ER affected genes in tumors. A continuing study of these genes and their effects should provide further insights into the role of ER as a transcription factor and the ER-pathway in the process of tumorigenesis.

COVALENT MODIFICATIONS CONTROL ACTIVITY OF SHRs

As with many transcription factors, covalent modifications play an important role in the modulation of activity of SHRs. For example, the activity of ERα can be modified by its phosphorylation, acetylation, ubiquitination, and palmitoylation. Several phosphorylation sites have been identified in ERα. Majority of these sites are in the activation function 1 (AF1). Serines at positions 104 and/or 106 are phosphorylated by CDK2 [Rogatsky et al., 1999]. Similarly, serine118 is phosphorylated by mitogen activated protein kinase (MAPK) [Kato et al., 1995], resulting in a ligand-independent transactivation of ER. Serine 118 is also phosphorylated by basal transcription factor TFIIH associated kinase CDK7 in a MAPK-independent and estrogen-dependent manner [Joel et al., 1988]. Serine 167 of ER is phosphorylated by casein kinase II and pp90rsk1 [Joel et al., 1998]. The identification of the phosphorylation sites and the finding that their mutations dramatically reduced transactivation of ER [Ali et al., 1993; Le Goff et al., 1994] has helped define the mode of activation and functional importance of the AF1 domain. In addition to these sites, serine 236, which resides in the C-terminal zinc finger of DBD is phosphorylated by protein kinase A (PKA) and leads to inhibition of dimerization and DNA binding [Chen et al., 1999]. Tyrosine 537, which resides in helix 12 of the LBD, has been found to be important in regulation of ligand binding, dimerization, and transactivation. Experimental replacement of tyrosine 537 with other amino acids resulted in a constitutively active receptor and increased basal association with coactivators [Wies et al., 1996]. In fact, one of the few natural mutants of ERα is a mutant where tyrosine 537 is replaced by arginine [Zhang et al., 1997], and this mutant activates gene transcription in a ligand-independent manner and exhibits a partial resistance to the action of anti-estrogens, implying a role for Y537R in the hormone independent status of the cells. Another important phosphorylation site in the AF2 domain is Serine 305, which could be phosphorylated by p21-activated kinase 1 (Pak1) [Wang et al., 2002], and phosphorylation at this site augments transactivation function of ERα. Mutation of serine 305 to alanine (S305A) abolished the stimulation by Pak1 and mutation to glutamic acid (S305E) to mimic phosphorylation resulted in an activated ER, validating the significance of Ser305. More recently, the Serine 305 site was also found to be phosphorylated by PKA, which bestowed tamoxifen resistance. On phosphorylation at this site, tamoxifen bound to the ER but failed to induce the inactive conformation and resulted in persistent ER transactivation [Michalides et al., 2004]. In brief, identification of different phosphorylation sites on ERα has revealed the functional importance of covalent modification in modulating the activity of the ER.

Acetylation is another covalent modification of ERα that affects its activity and hormone sensitivity. ERα is acetylated on lysine 303 in the hinge/LBD region by p300 [Wang et al., 2001], and this modification reduces the sensitivity of ER to the ligand. The clinical significance of lysine 303 acetylation was revealed by the natural occurrence of the Lys-to-Arg [K303R] substitution mutation in Caucasian women with breast hyperplasia [Fuqua et al., 2000]. This mutation, which was observed in 34% of Caucasian patients, resulted in ER that could not be acetylated leading to remarkable ligand hypersensitivity with a saturating response at physiological levels (10^{-11} to 10^{-12} M) of estrogen. In MCF-7 cells expressing this mutant form of ERα upon treatment with 10^{-12} M of estrogen, the proliferation rate was comparable to the rate with highest concentration of the hormone (10^{-9} M), indicating a drastic increase in response to estrogen by this mutation. This also suggests that the occurrence of this gain-of-function mutation had a pathological role in the onset of breast cancer [Fuqua et al., 2000]. More recently, ERα has been shown to be palmitoylated at cysteine 447 in the LBD region. ERα palmitoylation stimulates membrane localization of ER, promotes interaction with membrane protein caveolin-1 and activates nongenomic ER signaling, leading to an increased proliferation of cells [Acconcia et al., 2005].

Acetylation also constitutes a major regulating modification of androgen receptor (AR), which plays a key role in the progression of prostrate cancer. AR is acetylated by p/CAF, p300, and Tip60 [Fu et al., 2000; Gaughan et al., 2002]. AR acetlyation sites are located in the hinge region and are well conserved across species. In this context, it is important to point out that acetylation of ER reduces ligand sensitivity, whereas acetylation of AR augments ligand-induced activation [Fu et al., 2004]. This is one of several examples that highlight the fact that in spite of fundamental underlying functional similarities among SHRs, there is also significant difference in the manner in which each SHR is modulated. This advocates an in-depth study of each SHR to understand its specific role in development, proliferation, and tumorigensis in specialized tissues and organs.

COREGULATORY PROTEINS CONTROL SHR FUNCTIONS

The binding of the hormones to the respective SHRs is the first step in transmitting the signal carried by the hormone. The signal is further propagated by the SHRs DNA-binding capability, which occurs at specific gene targets, and transcription of the genes. The transcriptional upregulation of the target genes is achieved by the capability of the ligand-bound SHRs to recruit a wide bevy of proteins referred to as 'coactivators'. These coactivators have specialized functions of modifying the state of chromatin, recruitment of transcriptional machinery, and facilitating the transcription of the genes. Numerous families of coactivators of different SHRs have been identified. Their mode of interaction with the receptors and the molecular mechanism by which they function has been established. The ligand-binding domain of SHRs is structurally similar across the family. It is basically composed of 10–12 alpha helices that fold into an anti-parallel helical sandwich consisting of a central core of helices with helix bundles on either side [Wurtz et al., 1996]. This three-layered structure creates a wedge-shaped hydrophobic cavity where the ligand binds. The C-terminal-most helix (helix 12) extends away from the LBD core in the receptor when the ligand is not bound. Ligand binding induces conformational change, involving the repositioning of helix 12, which repositions itself by folding against the core of the LBD. This results in the sealing of the ligand binding cavity and the creation of a hydrophobic groove on the LBD, which is specifically recognized and bound by coactivators. The coactivators can bind to these hydrophobic pockets by the virtue of conserved motifs referred to as Nuclear receptor boxes (NR boxes), which are conserved motifs of five amino acids, 'LXXLL' (where L is Leucine and X is any amino acid). The motif forms an amphiphathic helix with the leucines presented outwards and involved in binding to the hydrophobic patch on the LBD of the ligand bound receptor. The mode of binding of NR box helices to the LBD of SHRs and the specificity of interaction have been extensively studied by X-ray crystallographic and mutational analysis and reviewed in detail elsewhere [Savkur and Burris, 2004].

STEROID RECEPTOR COACTIVATOR (SRC) FAMILY IN HORMONE ACTION

The first NR coactivator, steroid receptor coactivator-1 (SRC-1) was cloned by a yeast two- hybrid screen of the human B-cell cDNA library using the PR-LBD as bait [Onate et al., 1995]. It was found to interact in a ligand dependent manner with AF2 domain of various NRs like progesterone receptor (PR), ER, thyroid receptor (TR), retinoid-X-receptor (RXR), glucocorticoid receptor (GR), and peroxisome-proliferator-activated receptor (PPAR) and enhance their transcriptional activation. The second member of the family, SRC-2, also called as glucocorticoid receptor-interacting protein 1 (GRIP1), transcriptional intermediary factor 2 (TIF2)/nuclear receptor coactivator-2 (NCoA2) was identified as a protein binding to AF2 domains of ER and GR [Hong et al., 1996; Voegel et al., 1996]. The third member of this family was simultaneously identified and reported by several groups as retinoic acid receptor (RAR) -interacting protein (RAC3), a CBP-interacting protein (p/CIP), a hRARβ-stimulatory protein (ACTR), a gene amplified in breast cancer (AIB1) and as TR-interacting protein (TRAM1). P/CIP was the mouse homolog whereas RAC3/ACTR/AIB-1/TRAM was the human isoforms [reviewed in Leo and Chen, 2000]. A detailed study of the functioning of this family of coactivators has resulted in an in-depth understanding of the molecular mechanism by which SHRs and the NRs recruit the services of coactivators to enforce changes in the

transcription of the target genes as needed by hormonal signals.

The three members of the SRC family share about 50–55% sequence similarity. The most conserved region is the N-terminal region, which is referred to as the basic helix-loop-helix (bHLH) domain involved in the DNA binding and protein-protein interaction [Huang et al., 1993]. The central region has three NR boxes with LXXLL motifs involved in the interaction with the ligand bound nuclear receptors [Chen et al., 1997; Torchia et al., 1997]. In addition, there are two transcriptional activation domains (AD1 and AD2) in the C-terminal region. The AD1 region is responsible for interaction with the general transcriptional cointegrators, CBP and p300. This region has NR box motifs, and mutation of these NR boxes impairs the interaction of SRCs with the transcriptional regulators, implicating the importance of AD1 in recruiting acetyl transferases like CBP/p300 and p/CAF for chromatin remodeling [Voegel et al., 1998; Li et al., 2000]. The other activation domain, AD2 was found to be responsible for interacting with and recruiting histone methyltransferases and coactivator associated arginine methyltransferase 1 (CARM1) [Chen et al., 1999; Koh et al., 2001]. In brief, SRCs function as coactivators by chromatin remodeling and by recruiting other coactivators.

Results from mechanistic studies have helped to clarify the step-by-step process of SRC involved transcription activation at the molecular level. Ligand bound activated SHR/NR recruites a preexisting complex of SRC with p300, pCAF (p300/CBP associated factor) and CARM1 to the chromatin resulting in acetylation and methylation of histones at specific sites [Chakravarthi et al., 1996; Shang et al., 2000; Li et al., 2003]. Subsequently, the SWI/SNF (switch defective/sucrose nonfermenter) chromatin-remodeling complex is recruited through direct or indirect interactions with CBP/p300 and this complex brings about histone acetylations resulting in opening of the chromatin [Huang et al., 2003]. In the next steps the VDR-interacting protein or TR-associated protein (DRIP/TRAP) mediator complex is recruited by interactions with SRC/CBP/p300 complex or direct interactions with the NR. The TRAP complex directly interacts with the basal transcriptional machinery, which results in the initiation of gene transcription [Ito and Roeder, 2001; Sharma and Fondell, 2002; Huang et al., 2003].

In addition, several other coactivators have been identified such as steroid receptor RNA activator (SRA/SRAP), which is a unique coactivator of SHRs like PR, ER, GR and AR. SRA/SRAP existed as a ribonucleoprotein complex containing SRC-1 and it activated transcription by interacting with the AF1 domain of the SHRs, distinguishing it from other coactivators [Lanz et al., 1999]. E6-associated protein (E6-AP) is an ubiquitin protein ligase that was found to potentiate ligand-dependent transcription of ER, PR, AR and GR [Nowaz et al., 1999]. It is overexpressed in mammary tumors [Sivaraman et al., 2000]. L7/switch protein for antagonists (L7/SPA) is an antagonist-specific coactivator with a basic leucine zipper domain; it potentiates only the partial agonist activity of antagonists, including tamoxifen but not of the agonist [Jackson et al., 1997]. The PIAS family (protein inhibitor of activated signal transducer) is composed of a group of related proteins. The first member of the family (PIAS1) was found to be a coactivator of AR, PR, GR and ER [Kotaja et al., 2000; Tan et al., 2000] and it is overexpressed in 33% of prostate cancer samples, indicating a possible role in tumorigenesis of the testis or prostate [Li et al., 2002]. SNURF (small nuclear ring finger protein) was identified as an interacting protein of the DBD of AR and was subsequently found to be a coactivator of AR, GR and PR [Moilanen et al., 1998]. ARAs (Androgen receptor associated proteins is a large group of AR-interacting proteins of different molecular weights (ARA70, ARA160, ARA54, ARA267) which can bind and modulate the transcriptional function of AR [Yeh and Chang, 1996]. The identification of potentiators of the SHR activity has resulted in a wealth of information regarding the role of these positive coregulators in modulating the activity of the SHRs and their possible role in tumorigenesis.

Another SHR coactivator whose expression levels and activity in cancer has been found to be of great significance is SRC-3 or AIB1. It was found to be a cancer amplified coactivator and plays very important roles in biological processes involving cell proliferation, migration, differentiation, sexual maturation, and reproductive functions, as well as in breast cancer. SRC-3 was identified by several laboratories using different strategies [Lan et al., 2003]. It had functional domains characteristic to the SRC family of coactivators and could form stable complex with CBP to enhance transcription by

several nuclear receptors namely, RAR, ER, TR and PR. Altered expression levels of SRC-3 were seen in hormone-dependent cancers like breast cancer. SRC-3 mRNA was overexpressed in 31–64% of breast tumors [Anzick et al., 1997; Bouras et al., 2001]. Overexpression was seen in both ER-positive and negative tumors [Bautista et al., 1998]. The SRC-3 protein levels were not always correlated with the incidence of cancer. In one of the studies, a comparison of the normal tissue to the breast tumor showed high levels of SRC-3 protein in only 10% of the tumors [List et al., 2001]. In invasive breast tumors the overexpression of SRC-3 was correlated to high levels of human epidermal growth factor 2 (HER2/neu) [Bautista et al., 1998; Bouras et al., 2001]. High levels of AIB1 were also associated with tamoxifen resistance and worse survival-rate. Patients with high levels of both SRC-3 and HER2 have worse response to antiestrogen therapy [Osbourne et al., 2003]. Several studies have established the importance of AIB1 in cancers. Depletion of SRC-3 levels in MCF-7 breast cancer cells resulted in reduction of estrogen mediated cell proliferation and colony formation in soft agar assay [List et al., 2001]. In breast cancer cells AIB1 was recruited to cyclin D1 promoter, resulting in an increase of cyclin D1 expression [Planas-Silva et al., 2001]. A wealth of information was also obtained by the study of the SRC-3 knockout mice (SRC-$3^{-/-}$) harboring the *MMTV/v-Ha-ras transgene* (*ras* +). Virgin SRC-3 knockout mice had a lower incidence and extent of mammary gland ductal hyperplasia when compared to the wild type mice indicating that the initiation of tumorigenesis by *MMTV/v-Ha-ras oncogene* was suppressed in the knockout mice. The frequency of breast tumors and metastasis to the lungs was also considerably decreased in SRC-3 knockout mice. A similar pattern of tumor formation and metastasis was also found in mice through multiple pregnancies [Sinn et al., 1987]. These findings show the involvement of SHR coactivators like SRC-3 in the initiation and progression of cancers.

PELP1, A NOVEL COREGULATOR OF HORMONE ACTION

PELP1 has been identified as a novel ER coactivator. It is a protein with 1273 amino acids and unusually rich in prolines (13.2%), glutamic acid (12.4%) and leucine (12.9%), and hence, was named as PELP1 (Proline, Glutamic acid and Leucine rich protein) [Vadlamudi et al., 2001]. It has nine NR box motifs, seven towards the N-terminal region and two in the central region. It interacts with and significantly enhances the transcriptional function of ERα indicating that it functions as an ER coactivator. PELP1 did not have the same effect on PR and GR indicating that it may functional as an ER-specific coactivator [Vadlamudi et al., 2001]. Even though it has a molecular weight of 160 kDa, sequence analysis showed it was distinct from the other members of the p160 family of coactivators. The conserved domains of p160 proteins like the bHLH, PER, ARNT and SIM homology domains were absent in PELP1. It could interact with transcriptional activators like CBP and p300 *in vivo*, which suggest that PELP1 activates ER transcription by recruiting general coactivators like CBP and p300.

A possible role of PELP1 in mammary tumorogensis was also evident by the fact that a small number of breast tumors analyzed contain a 3–5 fold higher levels of PELP1 in comparison to normal adjacent tissue [Vadlamudi et al., 2001]. Subsequent studies suggested that PELP1 is identical to another ERβ interacting protein namely modulator of non-genomic activity of estrogen receptor (MNAR) [Wong et al., 2002]. The cDNAs of these two proteins localized to the same region of the chromosome i.e. 17 p13.3 and showed 99% sequence homology. In transient transfection assays MNAR also activated ERα transactivation function just as PELP1 does. MNAR was found to play a role as an activator of the non-genomic effects of ERα by stimulating the activation of the Src/MAPK pathways. The interaction of ER with Src was stabilized by MNAR and such events were identified as the mechanism with which MNAR activates the non-genomic effects of ER [Barletta et al., 2004].

A recent study found that the status of PELP1 could also affect cell cycle progression, as PELP1 associates with pRb (retinoblastoma protein), a cell cycle switch protein that is known to play a fundamental role in the proliferation, development, and differentiation of eukaryotic cells [Balasenthil and Vadlamudi, 2003]. PELP1 overexpression hyper-sensitizes breast cancer cells to 17β-estradiol (E2) signaling, enhances progression of breast cancer cells through the S phase of the cell cycle, and leads to persistent hyperphosphorylation of pRb in

an E2-dependent manner. These observations suggest that PELP1 plays a permissive role in estrogen mediated cell cycle progression via its interactions with the pRb pathway. Upon stimulation of cells with estrogen, there was an enhanced recruitment of PELP1 to the estrogen responsive promoters and colocalization with the acetylated histone H3. Increased levels of PELP1-associated acetlytransferase activity were observed on E2 stimulation. In the same study, PELP1 was also found to interact with histones H1 and H3, with a greater binding affinity to H1. It was found that PELP1 increases transcription by chromatin modification involving the displacement of H1 [Nair et al., 2004]. In another study, PELP1 was confirmed to act as ER coactivator but as a corepressor of GR and of non-NR sequence-specific transcription factors tested, including AP1 (activating protein 1), NF-κB (Nuclear factor-kappa B), and TCF/SRF (ternary complex factor/serum response factor) [Choi et al., 2004]. The noted repression activity of PELP1 was due to its ability to recruit HDAC2, which in-turn might mask the acetylation of histones H3 and H4 and, prevent them from acting as substrates for histone acetyltransferases. Ligand binding to ER reverses the repressor role of PELP1 with a parallel increase in the status of histone hyperacetylation. Recently it was also shown that PELP1 also functions as a coactivator of signal transducers and activator 3 (STAT3), increasing its transcriptional activity [Manavathi et al., 2005]. This positive regulation is brought about by the ability of PELP1 to augment growth factor induced phosphorylation of serine 727 of STAT3 via activation of src-MAPK pathway. STAT3 is proven to play a role in tumorigenesis and constitutive phosphorylation at serine 727 has been observed in several tumors. Additionally, genes such as *cyclin D1*, *c-myc*, and *c-fos*, which are involved in cell proliferation and oncogenesis, are targets of STAT3 [Levy and Darnell, 2002]. Functioning as a coactivator of STAT3 might be one of the ways in which deregulation of PELP1 activity or expression might be involved in tumorigenesis.

Another notable property of PELP1 is its ability to function as a coactivator of both ERα and ERβ in endometrial cells and potentiate the agonist activity of tamoxifen in endometrial cells but not in breast cells. PELP1 expression and localization was widely deregulated in the endometrial tumors [Vadlamudi et al., 2004]. Since PELP1 was primarily localized in the cytoplasmic and/or nuclear compartment in endometrial cancers, these studies provided important clues about the emerging cytoplasmic or non-genomic functions of PELP1. In brief, these studies revealed several facets of PELP1's role as a coactivator and corepressor of NRs and that PELP1 modulates both the genomic and nongenomic functions of ER. A continuing investigation of well-studied coactivators like SRC-3 and newly emerging members like PELP1 would further help in identification and clarification of the role played by these coregulator proteins in cancers and will result in establishing them as targets in prevention and treatment of cancers.

NON-CLASSICAL MECHANISM OF ER ACTIVITY

In addition to the classical mechanism of binding of SHRs to their response elements and altering the gene transcription, SHRs like ER were capable of altering gene expression without directly binding to the DNA. This was evident by the fact that about one third of human genes that are regulated by ER do not have the ER responsive (ERE) consensus sequences [O'Lone et al., 2004]. This is possible because ER binds and modulates the functions of other classes of transcription factors such as activator protein-1 (AP-1) or Sp-1, which in turn bind to their cognate response elements on the DNA [Gottlicher et al., 1998]. This phenomenon observed in the action of several NRs is referred to as 'transcriptional cross talk.'

In addition to genomic effects, steroid hormones can rapidly manifest their activity in cells in a manner that cannot be accounted for by the activation of RNA and protein synthesis. These effects are believed to be initiated by membranous and/or cytoplasmic receptors, and generally referred to as the 'Non-genomic' effects of the steroid hormones and are frequently credited to the activation of several protein-kinase cascades [Losel and Wehling, 2003]. In ER, these nongenomic effects are brought about by a sub-population of the classical ERs (ERα and ERβ), which localize at the plasma membrane [Pappas et al., 1995]. A truncated form of ERα was reported as the predominant form of ER at the plasma membrane of endothelial cells [Li et al., 2003]. Several functions of ER have been attributed to nongenomic actions including mobilization of intracellular calcium [Improta-Brears et al.,

1999], stimulation of adenylate cyclase activity, and production of cAMP second messenger [Aronica et al., 1994]. One of the most important pathways activated by the nongenomic functions of ER is the MAPK signaling pathway, which has been studied in great depth in cell types like breast cancer [Migliaccio et al., 1996], endothelial [Chen et al., 1999], and bone [Endoh et al., 1997]. MAPK is known to activate phosphotidyl inositol (PI) 3 kinase pathways in endothelial, breast cancer, and liver cells. As part of their nongenomic effects, membrane ERs also activates membrane tyrosine kinase receptors in various cell types. For instance, ligand activated ERα activates the epidermal growth factor receptor (EGFR), which involves the activation of G proteins, Src kinase, and matrix metalloproteinases, resulting in increased MAPK and Akt activities [Bjornstrom and Sjoberg, 2005]. ERα also binds and activates insulin-like growth factor (IGF-I) receptor, which ultimately results in the activation of the MAPK pathways [Razandi et al., 2005]. Several instances of the synergistic nature of the genomic and nongenomic effects of ER have been observed to alter the expression of the target gene, such as cyclin D1. The cyclin D1 promoter has no ERE-like sequences but its stimulation by estrogen primarily involves ER interaction with the Sp1 transcription factor at the GC-rich promoter sequences [Castro-Rivera et al., 2001] and with the c-Jun/ATF-2 heterodimers at the variant CRE [Sabbah et al., 1999]. On the other hand, it has also been shown that activation of cyclin D1 gene occurs by the association of ERα with Src kinase and the p85α regulatory subunit of PI3-kinase in breast cancer cells [Marino et al., 2002]. Interestingly, both the ERs (ERα and ERβ) are also targets of MAPK phosphorylation, which modulates their activity, ultimately affecting their genomic functions. On the whole, the effects of the steroid hormones in cells are the cumulative results of their genomic and nongenomic effects involving activation of a myriad of protein kinase pathways, which in-turn will influence secondary genomic responses.

ESTROGEN RECEPTOR β, ANOTHER SIGNIFICANT PLAYER IN ESTROGEN SIGNALING

A second ER was identified a decade ago and named ERβ to distinguish it from the previously identified and extensively studied ERα [Mosselman et al., 1996]. Later it was cloned from rat prostrate [Kuiper et al., 1996], and then from mouse [Tremblay et al., 1997] and human [Ogawa et al., 1998]. The possibility that ERβ is an isoform of ERα was eliminated by the fact that two different genes coded them. ERα gene is located on chromosome 6 and ERβ gene on chromosome 14 [Enmark et al., 1997]. ERβ protein has 530 amino acids and is smaller than ERα, which has 595 amino acid residues. Human ERα and ERβ share structural homology in their domains. Comparison of the amino acid sequences of various domains between the two receptors showed that the activation function domain (A/B or the AF1 domain) was the least conserved with 30% sequence similarity and studies have shown that the AF1 domain of ERβ is not involved in modulating its activity [Hall and McDonnell, 1999]. The two receptors share a maximum homology of 96% in the DNA-binding domain (domain C), 36% homology of the hinge region (domain D), and 53% sequence homology in the ligand binding or the AF2 domain. Similar to ERα, ERβ also functions as a ligand-dependent transcription factor. A comparison of the ligand binding affinities of the two receptors showed that they both bind to 17β-estradiol with the same degree of affinity but show subtle differences in binding to other ligands. In comparison to ERα, ERβ binds with twice the affinity to antiestrogens like 4-hydroxy tamoxifen (4-OHT) and ICI 164,384, and with half the affinity to diethylsilbesterol (DES) and five times the affinity to phytoestrogen like genistein [Kuiper et al., 1997].

Several variants of ERβ resulting from insertions, exon deletions, or alternative splicing have been discovered. These variants can also bind ligands and mediate estrogen signaling [Paech et al., 1997; Cowley and Parker, 1999]. The most studied variant of ERβ is ERβcx, which is truncated at the C-terminus and is transcribed using an alternative exon 8 and has an additional 26 amino acids because of alternative splicing. It has a reduced binding affinity to 17β-estradiol and to the EREs [Ogawa et al., 1998]. The most interesting aspect of this variant is the fact that it heterodimerizes with ERα and inhibits its transcriptional activity [Pace et al., 1997; Pettersson et al., 2000] suggesting it may have an important role in neutralizing the activity of ERα and may contribute to phenomena such as hormone independence and tamoxifen resistance. Further

studies showed that other isoforms of ERβ such as ERβ1, ERβ2, and ERβ5 also have the same negative effect on ERα [Peng et al., 2003], indicating the importance of these isoforms in the overall estrogen signaling.

The discovery of an additional ER, ERβ forced a reappraisal of the understanding of the estrogen signaling in cells and the therapies being used to treat breast cancer. ERα and ERβ have contrasting effects on cell proliferation in breast cancer. The specific functions of ERβ in cancer are not very well known in comparison to the level of understanding of the role played by its counterpart ERα, but initial studies suggest that ERβ might have an inhibitory effect on cell proliferation. This concept was revealed by the fact that the levels of ERβ are highest in normal tissue as well as in benign disease but decrease during carcinogenesis [Brandenberger et al., 1998]. Accordingly, the ratio of ERα to ERβ was observed to increase during carcinogenesis [Pujol et al., 1998; Rutherford et al., 2000], strengthening the concept of ERα as promoting tumorigenesis while ERβ of preventing it. As additional proof is loss of the ERβ expression could be one of the leading events to breast and ovarian carcinogenesis [Lazennec et al., 2001; Bardin et al., 2004]. Mechanistic studies of the functions of ERβ are revealing the reason behind its anti-proliferative effects in contrast to ERα. One of the possibilities is the dominant negative effect the ERβ variants have on ERα by heterodimerizing with it and silencing its activation functions.

The two ERs differ distinctly in the mode of interacting with the coregulator proteins. For instance, in ERα the binding of the agonist leads to the release of corepressors like N-CoR and SMRT and binding of coactivators and transcriptional activation. ERβ has been shown, both in vitro and in vivo to bind the corepressor N-CoR and SMRT in the presence of ER agonists such as estradiol and phytoestrogens such as genestein but not in the presence of antiestrogens [Webb et al., 2003]. In MDA-MB231 cells, ERβ inhibited cell proliferation in a ligand-dependent manner, in contrast to the induction of cell proliferation by ERα [Lazennec et al., 2001]. In a recent study, stable cell lines of ERβWT and the variant ERβcx mutant in MCF-7 cells showed reduced the percentage of cell population in the S-phase and the number of colonies in the anchorage independent assay [Omoto et al., 2003]. In another study, induced expression of ERβ in ERα-positive breast cancer cells led to inhibition of their growth. It was also found that this reduction in proliferation by ERβ might be due to repression of the cyclin D1 gene, which is a key player in controlling the G1–S transition and thus cell proliferation. Numerous other components of the cell cycle involved in proliferation such as cyclin E or Cdc25A were decreased [Strom et al., 2004]. Increased cell proliferation and response to estrogen in ERβ-knockout mice suggests that it plays a role in the modulating the effects of ERα. The antiproliferative effects of ERβ are also evident in both prostate and colon tissue. ERβ is expressed in high levels in the prostate and colon when compared to ERα. It was shown in the knockout mice that ERβ is involved in the regulation of epithelial cell growth and its absence resulted in prostatic epithelium [Weihua et al., 2001]. A distinct loss of ERβ was observed in colon cancer cells, but the mRNA levels did not change [Foley et al., 2000]. A change in the localization of ERβ was also observed in colon cancer cells where it was found to be cytoplasmic, whereas in the normal cells it was predominantly nuclear [Witte et al., 2001]. Several lines of evidence from studies in various tissues point towards the possibility of ERβ functioning as a tumor suppressor. Most importantly ERβ has an antiproliferative effect and antagonizes the functions of ERα advocating continuing research of this receptor as a target for cancer prevention and treatment.

THERAPEUTIC APPROACHES FOR HORMONE DEPENDENT CANCERS

Extensive research on the molecular mechanisms involved in the action of steroidal hormones has helped to identify potential molecular targets in cells that could be modulated to prevent and treat cancer. As most of the action of the hormones occurs via their receptors, it is not surprising that a majority of drugs being used are compounds, which modulate the functions of these receptors. Because of the complexity involved in the action of the steroidal hormones and the wide array of effects these receptors exert in cells, it has been a challenge to formulate drugs with maximal beneficial effects with a minimal and preferably no undesirable side effects. In the case of breast cancer, a class of drugs generally referred to as selective ER modulators or SERMs, which act as

receptor binding competitors of estrogens and block the effects of estrogens, have been used as therapeutic agents. One of the most widely and successfully used drugs is Tamoxifen, which is a nonsteroidal antiestrogen that antagonizes the action of estrogens and is being used in both prevention [Fisher et al., 1998] and treatment of breast cancer [Cole et al., 1971]. It is a very efficient SERM in the breast tissue, but it had a different mode of action in other tissues. For example, in endometrium and bone tissues, tamoxifen acts as an agonist increasing the risk of endometrial cancer in patients [Fisher et al., 1994]. In addition to this, prolonged administration of tamoxifen has resulted in development of resistance to its effects and due to its slightly 'agonistic' effects even tumor regrowth has been observed [Johnston, 1997]. Once the fallouts of prolonged tamoxifen use were realized, efforts were accelerated to synthesize new versions of tamoxifen with improved agonist and antagonist effects. This has led to the synthesis and testing of several other analogues such as toremifene, iodoxifene, droloxifene, and TAT-59. These compounds also show mixed tissue dependent agonist and antagonist activities [Johnston, 2005].

At the molecular level, SERMs serve as antiestrogens that bind to the ligand-binding domain (AF2) of the ER and because of their different molecular structures; they induce a conformational change in the LBD that is different from the change brought about by estrogens. This altered conformation prevents the coactivators from binding to AF2 blocking the transactivation function of the receptors. As they affect only the AF2 domain, the AF1 domain is not affected and still can bring about transcriptional upregulation of the target genes. The effect of the AF1 domain has been attributed to the partial agonist action elicited by SERMs. A constant search for a perfect or pure antiestrogen with no agonist activity and a higher potency has resulted in the discovery of another class of drugs referred to as 'selective ER downregulators' (SERDs), one of the most widely used member of which is fulvestrant (ICI 182780), a steroidal anti-estrogen. The mode of functioning of this family is to bind to the ligand-binding domain and by their long bulky side chains at the 7α and 11β the affect dimerization of ER, resulting in the lack of binding of the receptor to DNA, higher receptor degradation and hence, absolutely no agonist effect [Parker, 1993]. Clinical trials suggest fulvestrant as an effective drug as compared to tamoxifen. It has 100-fold higher affinity to ER and no agonist activity in the uterus. It can completely block the stimulatory activity of both estrogens and partial agonist activity of SERMs [reviewed in Howell et al., 2000].

In addition to targeting the functioning of the receptors, another effective approach for treatment of breast cancer is to inhibit the estrogen synthesis. Even though the ovaries are the principal source of estrogen in premenopausal and non-pregnant women, other sites of estrogen biosynthesis are present throughout the body. These sites, which include the mesenchymal cells of the adipose tissue and skin [Simpson et al., 1997], osteoblasts [Bruch et al., 1992], vascular endothelium [Sasano et al., 1999], and aortic smooth muscle cells [Naftolin et al., 1975], are the major sources of estrogen in postmenopausal women. The estrogen synthesized in these sites is biologically active at the local tissue level in paracrine or intracrine fashion. These sites of estrogen synthesis are capable of increasing local levels of estrogen to considerable high levels, which plays an important pathophysiological role in carcinogenesis. In view of these findings, current strategies are designed to inhibit the local synthesis of estrogens. One of the important therapeutic approaches is to inhibit the enzyme involved in the biosynthesis of estrogen, namely aromatase cytochrome P450 or estrogen synthetase and the class of inhibitors, which are being used, are referred to as aromatase inhibitors (AIs). These inhibitors are being exclusively used in postmenopausal women as these agents have little ability to obliterate aramatase activity in premenopausal ovaries [Simpson and Dowsett, 2002]. Aminoglutethimide was the first approved AI and has been used as a drug for several years in breast cancer therapy [Santen et al., 1982; Santen and Harvey, 1999]. This first generation AI was non-specific and could involve several enzymes, which are involved in estrogen biosynthesis. This can result in the inhibition of estrogen synthesis in a global fashion and could have detrimental side effects at sites where estrogen is essential for normal functioning such as bone mineralization and maintainance of cognitive functioning in the brain. Later generations of AIs with higher potency and specificity such as anastrazole, letozole, and exemestane, are US Food and

Drug Adminstration (FDA) approved for treatment of advanced breast cancer are currently reaching culmination of their clinical trials. In the view of the fact that the most extensively used breast cancer drugs like tamoxifen and other SERMs are not generally potent for an indefinite period of time, the AIs have the potential of providing an alternate treatment either as back-up drugs or even first-line drugs for breast cancer treatment.

SUMMARY

In the present review, we have provided an overall view of the functions of the SHRs by focusing on the actions of estrogen via its receptors, which remains one of the most well understood and researched steroid pathways. We have elaborated on a wide variety of factors that affect this pathway, with special emphasis on the classical or genomic effects that are responsible for a major portion of estrogen effect. We also have discussed in detail the modulation of the functioning of the ERs by the coregulatory proteins, specifically the coactivators. A detailed description on the functions of a well-studied SHR coactivator, AIB1 or SRC-3, and a relatively new ER coactivator namely PELP1 has been given to provide an insight into the functioning of these proteins. A detailed analysis of the second ER, ERβ has been undertaken as it is emerging as a vital player in estrogen signaling by its transcriptional functions and the ability to affect the functions of ERα. To maintain the brevity of the review discussion on the role of the corepressor family of coregulators has been excluded. A brief discussion on the 'nongenomic' pathway of estrogen functions has been included. We also have briefly discussed the use of therapeutic agents, which have, emerged from the understanding of the role played by estrogens and its receptors in normal and malignant events in the target tissues. A schematic diagram has been provided reflecting the different modes of action and complexity of the functioning of estrogens and its receptors (Fig. 1). There lies an underlying commonality in the functioning of the

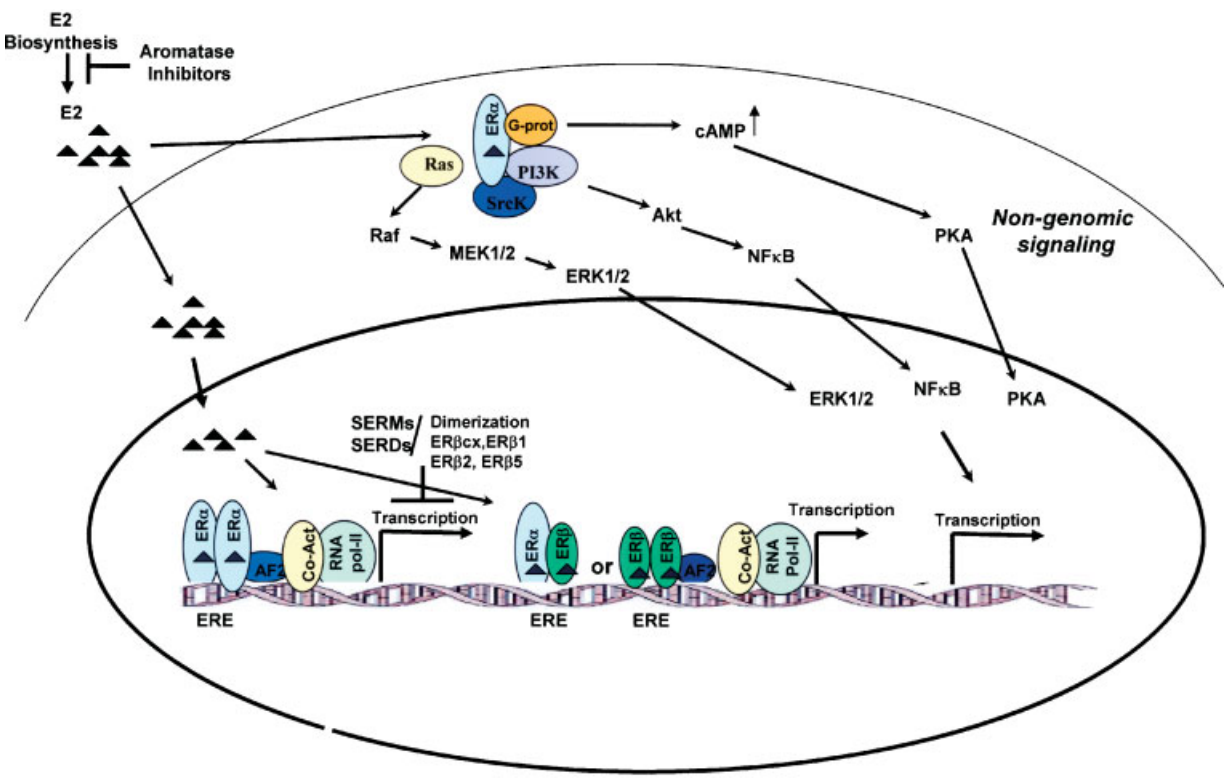

Fig. 1. Receptor mediated estrogen-signaling pathways. A schematic diagram depicting the complexity of estrogen signaling in the cells. The genomic and nongenomic effects are highlighted along with the therapeutic approaches being used.

steroid hormones and their respective receptors. This is evident by the considerable degree of similarity in the functioning, adverse effects of deregulation and therapeutic approaches that have been observed in the case of steroidal hormones. Extensive studies have also revealed that they draw from a common pool of coactivators and corepressors to modulate their functions. An appreciable degree of deviation and differences have also been revealed between the steroidal hormone actions that validate continuing research on each one of them. Further research on steroidal hormones, their receptor functions, and the different pathways by which they manifest their effects would help in better recognizing their roles in the normal functioning of cells and would help in the development of more effective therapeutic approaches to prevent and treat the tumorigenic effects that result from their deregulated activity.

ACKNOWLEDGMENTS

We thank the members of Dr. Kumar's laboratory for their helpful discussions. We apologize to several of our colleagues for not citing their primary references because of space limitation.

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