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Molecular Mechanisms of Estrogen and Antiestrogen Action

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Abstract.

Estrogen is a key regulatory hormone that affects numerous physiological processes. The estrogen receptors (ER), ERα and ERβ, play a central role in mediating the effects of both estrogens and antiestrogens. Estrogen signaling has proven to be multifaceted and the precise mechanism of action remains in many cases elusive. The work presented in this thesis provides further insight into the mechanisms that control the transcriptional regulation of estrogen and antiestrogen responsive genes.

The effect of tamoxifen and raloxifene on the sex hormone binding globulin (SHBG) gene was studied in human hepatocarcinoma cells (HepG2). Both tamoxifen and raloxifene functioned as estrogen agonists on the SHBG gene at doses higher than 1μM in a non-ER-dependent fashion. Both compounds displayed an additive effect to estrogen induced ER-dependent SHBG expression. Thus, we propose that the elevated SHBG levels observed in pre-menopausal women on adjuvant tamoxifen treatment may be explained by non-ER dependent tamoxifen agonism.

The discovery of ERß promises an opportunity to develop ligands with improved tissue selectivity. Despite the great homology of the amino acids lining the ligand-binding cavities of ERα and ERß, we showed selective effects of ERα and ERß on an ERE-reporter in response to a set of ER-ligands. We showed that tamoxifen, 4OH-tamoxifen and raloxifene displayed an ERα-selective partial agonism, whereas their effect via ERβ was antagonistic. In conclusion, our data indicated that it is possible to develop novel receptor subtype specific ligands that may have an improved tissue selectivity and side effect profile.

The pS2 gene is estrogen responsive in hepatocarcinoma cells (HepG2) in the presence of ERα (HepER3 cells). The estrogenic activity is mediated through an estrogen response element (ERE) in the 5´-flanking region of the pS2 gene, however, an AP1 response element located close to the ERE in the pS2 promoter was also essential for response to estrogen. The potentiation of pS2 promoter activity by the AP1 motif in response to estrogen was dependent on the ligand-binding domain of ERα. Furthermore, the presence of an intact AP1 element in the pS2 promoter sustained suppression of pS2 promoter activity by an LXXLL peptide. The phorbol ester PMA stimulated pS2 expression in both HepER3 and the parental, non-ER expressing, HepG2 cells although its activity was substantially less in HepG2 cells. The effect of PMA was mainly mediated through the AP1 element. In summary, the data suggest that the effect of estrogen is mediated through a crosstalk between the ERE and the AP1 response element and that ERα plays a crucial role in mediating the effect, not only of estrogen but also of PMA.

We show estrogen-induced synergistic activity by the p160 coactivator SRC-1, mediated via the ERE and the AP1 response element in the pS2 promoter. In addition, we present data that support an interaction between the ERE and the AP1 motif via SRC-1. Also TIF-2, a related but distinct p160 coactivator, potentiated the estrogenic response of the pS2 gene, however, TIF-2 was less dependent on an intact AP1 response element in the pS2 promoter than SRC-1. Furthermore, the type of ERE in the pS2 promoter influenced the potentiation by SRC-1; in support of this, there was less dependence on the AP1 motif when the natural ERE was substituted for by a consensus ERE. These results highlight several mechanisms whereby fine-tuning of estrogen responsiveness of an individual gene may be achieved.

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Main References

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Barkhem, T., Andersson-Ross, C., Höglund, M. and Nilsson, S. (1997). Characterization of the "estrogenicity" of tamoxifen and raloxifene in HepG2 cells: regulation of gene expression from an ERE controlled reporter vector versus regulation of the endogenous SHBG and pS2 genes. *J Steroid Biochem. Molec. Biol* 62:53-64

II. Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J-Å. and Nilsson, S. (1998). Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists *Molecular Pharmacology* 54: 105-112

III. Barkhem, T., Haldósen, L-A., Gustafsson, J-Å. and Nilsson, S. (2002). pS2 gene expression in HepG2 cells: complex regulation through crosstalk between the estrogen receptor α, an estrogen-responsive element, and the activator protein 1 response element. *Molecular Pharmacology* 61: 1273-1283

IV. Barkhem, T., Haldósen, L-A., Gustafsson, J-Å. and Nilsson, S. Transcriptional synergism on the pS2 gene promoter between a p160 coactivator and ERα depends on the coactivator subtype, the type of estrogen response element, and the promoter context. *Molecular Endocrinology (in press).*

Contents.

Abbreviations.

3D	Three-dimensional
AF	Activation function
AP1	Activator protein 1
BERKO	Estrogen receptor β knock out
CBP/ p300	CREB-binding protein/ p300
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CVD	Cardiovascular disease
E2	17β-estradiol
EGF-1	Epidermal growth factor-1
ER	Estrogen receptor
ERE	Estrogen response element
ERKO	Estrogen receptor α knock out
GRIP-1	Glucocorticoid receptor interacting protein 1
H1-H12	Helix 1- helix 12
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDL	High density lipoprotein
IGF-1	Insulin-like growth factor-1
LDL	Low density lipoprotein
LH	Luteinizing hormone
MAPK	Mitogen activated protein kinase
Mox	Moxestrol
N-CoR	Nuclear receptor corepressor
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NR	Nuclear receptor
NR-box	Nuclear receptor box
pCIP	p300/CBP cointegrator-associated protein
PR	Progesterone receptor
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
Ral	Raloxifene
SERM	Selective estrogen receptor modulator
SHBG	Sex hormone binding globulin
SMRT	Silencing mediator of retinoic acid and thyroid hormone
SRC-1	Steroid receptor coactivator-1
Tam	Tamoxifen
TIF-2	Transcriptional intermediary factor-2
TRAP	Thyroid receptor associated protein

Introduction.

In general, estrogen has been considered a female reproductive hormone, however, estrogens exert a wide repertoire of biological effects in different types of tissues not only in females, but also in males. In addition to the growth and developmental effects on the female mammary glands and urogenital tract, estrogens affect the cardiovascular system, the skeleton and the central nervous system. Furthermore, estrogens play a role in pathological conditions, e.g. breast- and endometrial cancer.

Estrogens are small and lipophilic hormones that are believed to enter target cells by passive diffusion whereafter the hormone binds to the estrogen receptor (ER), which is a ligand-induced transcription factor. In the absence of a ligand the ER is complexed with inhibitory proteins, thus, held in a latent state. Upon binding hormone, the receptor is transformed into an active state and may interact with regulatory sequences termed estrogen response element (ERE), within target genes, resulting in transcriptional activation. To date, this classical mode of ER action is relatively well understood, known to be influenced by a variety of different parameters. These include hormone-induced conformational changes of the ER structure and interactions with cofactors that modulate the activity of ER regulated genes. However, estrogen signaling has been shown to be multifaceted and includes many different mechanisms. In addition to the classical ER/ERE pathway, estrogen target genes may be regulated through crosstalk between ER and a variety of signaling pathways.

This work has focused on mechanisms that control the transcriptional activity of estrogen and antiestrogen regulated genes. The study provides insight into the complex regulation of natural estrogen responsive promoters, whose regulation may depend on multiple interactions between the ER and additional protein factors. Furthermore, evidence is presented that the transcriptional activity of estrogen and antiestrogen regulated genes can also be mediated via non-ER dependent mechanisms.
The existence of two different ER subtypes, with distinct tissue distribution, adds further complexity to estrogen signaling. This topic has been addressed by evaluating ER subtype selective responses to different types of ligands.

Nuclear Receptors.

The nuclear receptor (NR) superfamily constitutes a large group of structurally related transcription factors that are regulated through ligand activation but also contains several members, referred to as orphan receptors, for which there are no apparent ligands (Baulieu et al., 1990). NRs regulate a wide range of important biological processes in mammals such as sexual differentiation, development, metabolism and homeostasis. Furthermore, NRs are recognized as important targets for pharmaceutical drug development to a broad range of diseases.

Approximately 50 different NRs have been found in mammals. NRs appeared early in evolution and are thought to be evolutionarily derived from a common ancestor gene. To date, NRs have been found in species ranging from coral to man, whereas no NR sequences have been found in sponges, plants or yeast. Phylogenetic analysis has revealed six different classes of NRs with similar functional characteristics in terms of DNA binding specificity and dimerization preferences (Laudet, 1997). It has been proposed that the ancestral NR was a constitutive transcription factor and that ligand binding has been acquired during the course of evolution (Laudet, 1997). However, the nuclear receptor superfamily lacks a clear evolutionary pattern in relation to the presence or absence of a ligand. Neither is it possible to predict the chemical nature of a ligand, which suggests that the ligand binding ability was independently acquired in the different subgroups.

One large subgroup, commonly referred to as type II NRs, includes the thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR) and peroxisome proliferator-activated receptor (PPAR), which preferentially bind to DNA as heterodimeric complexes with the retinoid X receptor (RXR). In addition, the members of this subgroup constitutively bind to DNA in the absence of ligand.
The steroid receptors, referred to as type I NRs, i.e. the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the progesterone receptor (PR), the androgen receptor (AR) and the estrogen receptor (ER), constitute one subgroup. Also this subfamily includes orphan receptors, the ERRs for which no endogenous ligands yet have been identified, although synthetic ligands were recently reported (Coward et al., 2001; Tremblay et al., 2001a; Tremblay et al., 2001b). The steroid receptors lack insect homologes indicating that this subfamily is evolutionarily younger than other subgroups

of the nuclear receptor superfamily. The remaining subgroups of the nuclear receptor superfamily include three separate classes of orphan receptors and one subgroup that, among other members, contains the RXRs that play important roles in nuclear receptor signaling as they are dimerization partners for many different receptors.

Roles of nuclear receptors in transcriptional activation.

Regulation of expression of eukaryotic genes is a highly complex and finely tuned process that involves a plethora of different protein factors. Eukaryotic genes transcribed by RNA polymerase II require basal transcription factors that recognize core promoter sequences to properly target the RNA polymerase to the promoter. The core promoter may contain a TATA-box sequence, which is recognized by the basal transcription factor TFIID, composed of the TATA box binding protein (TBP) and several associated factors (TAFs). TFIID and many other basal transcription factors form together with RNA polymerase II a multiprotein complex that is required to initiate transcription. The pre-initiation complex was originally thought to be formed through stepwise recruitment of the different components, starting with the recruitment of TFIID. However, recently a pre-assembly model has been proposed, which suggests the recruitment of a pre-formed RNA polymerase II holoenzyme complex (Lemon and Tjian, 2000).

Sequence-specific transcription factors, e.g. the NRs, interact in general with target sequences located upstream of the core promoter; however, functional recognition sequences for various site-specific transcription factors are also found downstream of the core promoter (Krieg et al., 2001). The activity of complex natural gene promoters is often controlled by multiple transcription factors that bind to distinct regulatory elements within the promoter and mediate responses to different signal transduction pathways. The concerted action of different transcription factors can therefore integrate multiple extracellular signals at a specific promoter resulting in a transcriptional response. Thus, the transcriptional activity of NRs at complex eukaryotic promoters may depend on cooperative interactions with other sequence-specific transcription factors.

One role that has been ascribed to NRs in the regulated expression of eukaryotic genes is to interact, by direct or indirect means, with the basal transcription machinery, facilitating recruitment and assembly of the pre-initiation complex. Indirect interactions are likely to involve coactivator proteins that are associated with the liganded NR and form bridges extending to the basal transcription factors at the core promoter (Jiang et al., 1998; Swope et al., 1996). However, NRs may also interact directly with factors at the core promoter; ERα and PR (Ing et al., 1992) have e.g. both been reported to interact with TFIIB while ERα also interacts directly with TBP (Sadovsky et al., 1995).

Another role of the NRs in transcriptional activation of target genes seems to be recruitment of factors, e.g. coactivator complexes that actively disrupt the higher order chromatin structure of the gene promoter region and facilitate access of additional transcription factors. Several coactivators of NRs possess intrinsic histone acetyltransferase (HAT) activity (Chen et al., 1997; Ogryzko et al., 1996; Spencer et al., 1997). The complex of different types of coactivators is thought to acetylate lysine residues of the histones that constitute the nucleosome, probably neutralizing positively charged lysine residues, resulting in a weaker interaction of the histones with the negatively charged DNA. This provides a less compact nucleosomal structure that enables access of transcriptional activators to the promoter, resulting in transcriptional activation.

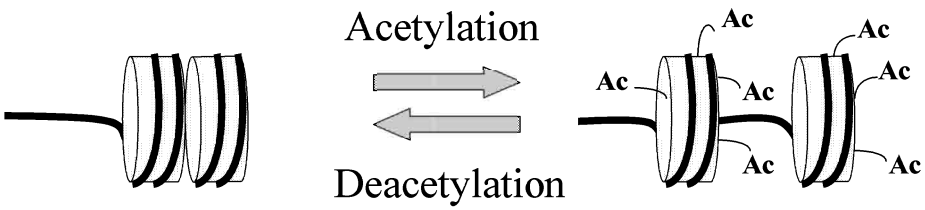


Figure 1. Acetylation of histone residues disrupts chromatin structure and allows transcription, whereas deacetylation of the histones results in dense chromatin structure and transcriptional repression.

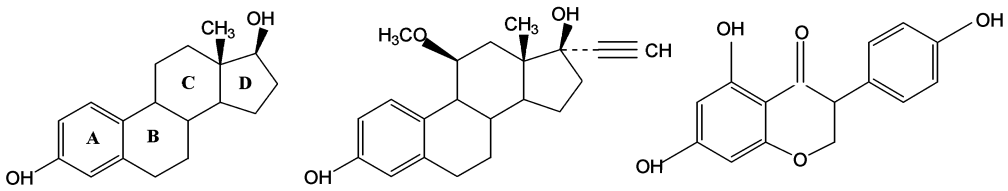
Estrogens.

Biosynthesis of estrogens.

The estrogens are 18-carbon steroids that are synthesized from the common precursor cholesterol. In the cytoplasm, cholesterol is bound to the sterol carrier protein 2. Another protein, the steroidogenic acute regulatory protein, transfers the cholesterol from the cytosol to the inner membrane of the mitochondrion, where the cytochrome P450 enzymes that catalyze the cleavage of the side chain of cholesterol are located. The last step in the process of estrogen synthesis is aromatization, which is catalyzed by a P450 aromatase present in the smooth endoplasmatic reticulum.

In addition to the endogenous estrogens, 17β-estradiol (E2), estrone and estriol, there are several compounds found in plants that have estrogenic properties, referred to as phytoestrogens. These include, for instance, the ERβ selective compound genistein (Barkhem et al., 1998), which is an isoflavonoid found in soybeans.

When studying estrogen-induced gene expression in liver cells (HepER3) we have preferred to use the synthetic estrogen analogue moxestrol rather than E2, since E2 is readily metabolized in these cells. Moxestrol exhibited the same characteristics as E2 in the HepER3 cells except for an approximately ten-fold higher potency.



17β-Estradiol Moxestrol Genistein

Figure 2. Molecular structure of estrogen receptor agonists.

Endogenous secretion of estrogens.

The secretory activity of steroid producing cells is closely linked to their biosynthetic activity since steroid hormones are not stored in steroidogenic cells. The most potent endogenous estrogen is E2, which is synthesized in the ovary under control of the hypothalamic-pituitary-gonadal axis. The primary sources of E2 in the ovaries are the theca and granulosa cells that according to a "two-cell theory" produce estrogen, whereby the theca cells secrete androgens that diffuse to the granulosa cells to be aromatized to estrogens (Hillier et al., 1994).

While most of the circulating E2 in premenopausal women results from direct ovarian secretion, aromatization of androgens in peripheral tissue is regarded as an important source of estrogen in males and in postmenopausal women. Aromatase activity has been detected in tissues such as muscle, fat, nervous tissue and the Leydig cells of the testes (Brodie and Inkster, 1993; Matsumine et al., 1986; Miller, 1991; Naftolin et al., 1975).

The two other major forms of endogenous estrogens, estrone and estriol, are both considerably less potent than E2. Estriol is produced in large amounts during pregnancy through a combined activity of the placenta and the fetus.

Transport and metabolism of estrogens.

When released into the circulation, the major pool of estrogens is bound to plasma proteins. In healthy women, the fraction of free E2 that is circulating in plasma is only about 2% of the total amount. About 60% is bound to albumin and about 38% is bound with high affinity to the sex hormone binding globulin (SHBG)(Dunn et al., 1981). The SHBG is a glycoprotein synthesized in the liver that also binds and transports testosterone and dihydrotestosterone (Westphal, 1986).

Hepatocytes are probably the major site of plasma SHBG biosynthesis (Khan et al., 1981), but the SHBG gene is also expressed in the Sertoli cells of testis where it is referred to as testicular androgen-binding protein. In testis the protein is differentially glycosylated and is thought to control androgen dependent sperm maturation by providing a high androgen concentration within the testis (Hammond, 1993). Human

plasma levels of SHBG are increased during pregnancy or by oral administration of estrogen or thyroid hormone but decreased by androgens (Anderson, 1974). Consequently, the serum level of SHBG in premenopausal women is twice as high as in men. Furthermore, the antiestrogen tamoxifen increases SHBG serum levels *in vivo*. Both pre- and postmenopausal women receiving adjuvant tamoxifen therapy for advanced breast cancer exhibit increased concentrations of SHBG in serum (Bruning et al., 1988).

Circulating E2 is rapidly converted in the liver to estrone and estriol which after sulfation or glucuronidation are excreted into the bile or urine. Estrogens are also metabolized by hydroxylation and subsequent methoxylation at the 2- or 4-positions of the A-ring.

The estrogen receptors.

The presence of an estrogen binding receptor protein was first reported in the early sixties by the pioneering work of Jensen and Jacobsen (Jensen and Jacobsen, 1962). The cDNA encoding the estrogen receptor (ER) protein was isolated and cloned in the middle of the eighties (Green et al., 1986; Greene et al., 1986) and was for long believed to be the only existing ER. However, recently an additional ER was discovered and cloned from rat prostate (Kuiper, 1996). The new receptor was designated ERβ and consequently the previous receptor was renamed ERα. Since the discovery of rat ERβ, human and mouse orthologs have also been cloned (Enmark et al., 1997; Mosselman et al., 1996; Pettersson et al., 1997; Tremblay et al., 1997). Human ERα is a protein of 595 amino acids, whereas human ERβ cDNA has been reported to encode a protein of 530 amino acids (Fuqua et al., 1999; Ogawa et al., 1998a). Recently, however, a human ERβ of 548 amino acids was described (Wilkinson et al., 2002). Both the mouse and the rat ERβ genes contain open reading frames that encode proteins of 549 amino acids. Alignment of the N-terminally extended region of the human 548 amino acid form of ERβ with the mouse and the rat ERβ proteins revealed that the proteins were highly homologous in this region.

Several differentially spliced forms of ERα have been reported (Fuqua et al., 1993; Murphy et al., 1998). These are, in particular, cDNAs isolated from breast cancer cells. Whether all differentially spliced mRNAs of ERα are translated into protein and play any biological role requires further investigations. Also for ERβ, different spliced variants have been identified. An insertion variant that contains an extra 18 amino acids between exon 5 and 6 was cloned from rat ovary (Chu and Fuller, 1997; Petersen et al., 1998). This cDNA encodes a protein that exhibits functional DNA-binding and dimerization properties, although it was shown to bind ligand with reduced affinity (Petersen et al., 1998). Subsequently, a similar cDNA has been found also in human cell lines (Hanstein et al., 1999). However, the human variant does not appear to exist as a protein due to an out of frame mutation (Eva Enmark, personal communication). Another ERβ variant, designated ERβcx, was found to be truncated at the C-terminus where the amino acids from exon 8 were replaced with 26 unique amino acids (Ogawa et al., 1998b). Thus, ERβcx lacks the AF-2 core region and was shown unable to bind ligand. Interestingly, ERβcx was shown to preferentially inhibit ERα induced expression of an ERE-driven reporter vector, presumably through heterodimerization with ERα. The physiological relevance of these ERβ splice variants remains, however, to be elucidated.

The tissue distribution of ERα and ERβ is, in part, different suggesting that the two receptors have distinct biological functions. For instance, ERβ appears to be the predominant receptor in the prostate, the bladder, the lung, the ovary, the CNS, the colon, the stomach, the heart and in the blood vessels (Couse and Korach, 1999; Dechering et al., 2000; Kuiper et al., 1997; Nilsson et al., 2001; Taylor and Al-Azzawi, 2000). However, within a particular tissue, the expression of a receptor subtype may be localized to a specific cell-type. For instance, the ovary shows high expression of both ERα and ERβ. A closer examination revealed that ERβ expression was restricted to the granulosa cells whereas ERα was expressed in the theca cells (Fitzpatrick et al., 1999). This suggests that ERα and ERβ may have distinct functions within a specific tissue.

ERα and ERβ domain structure.

NRs share a common modular structure that is made up of independent but interacting functional domains. These consist of the highly variable A/B domain in the N-terminal region of the receptor, a central conserved DNA binding domain (DBD or C-domain) through which the receptor contacts specific regulatory sequences in target genes. The D-domain is referred to as the hinge domain and serves as a linker region between the DBD and the relatively well-conserved ligand-binding domain (LBD or E-domain). Some NRs also contain a region (F-domain) that extends C-terminally to the LBD; no specific role, however, has been clearly assigned to this domain. Most NRs, including ERα and ERβ, are structurally organized according to these principles although there are atypical receptors, e.g. orphan receptors that lack a functional DBD (Giguere, 1999).

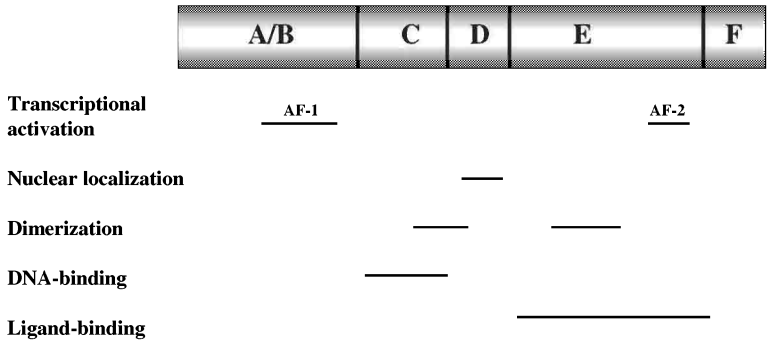


Figure. 3. Schematic view of ERα and ERβ domain structure

A/B domain.

The N-terminal A/B domain is the most variable region of the functional domains of the ERs and the human ERα and ERβ share less than 20% amino acid identity in this region, indicating that this domain may contribute to ER subtype specific action on target genes. This region is also less well conserved between different species as compared to any of the other functional domains of the receptors. The A/B domain harbors an activation function (AF-1) (Tora et al., 1989) that is ligand-independent and shows promoter- and cell-specific activity. Furthermore, the transcriptional activity of the A/B domain has been reported to be regulated through specific phosphorylation mediated by different signal transduction pathways.

DNA binding domain.

The central C-domain is the DNA binding domain. These domains of ERα and ERβ are highly homologous and share 95% amino acid identity. The DBD is structurally organized in two zinc finger motifs, which are involved in sequence-specific DNA-binding. These structures are conserved across the nuclear receptor superfamily. The structure of each zinc finger is stabilized by binding of a Zn^{2+} ion that is coordinated by four cysteine residues. The P-box [a sequence of 6 amino acids; CEGCKA (in the single letter amino acid code)] located in the C-terminal base of the first zinc finger contains amino acids that confer specificity for the estrogen response element (ERE). The P-box is identical in ERα and ERβ, thus, the two receptors can be expected to have similar DNA binding properties with respect to specificity and affinity.

The minimal target sequence on DNA recognized by the nuclear receptor DBD consists of a six base pair sequence referred to as a half-site. The consensus ERE is composed of two halfsites of the core sequence AGGTCA that are oriented in a palindromic order with a spacing of three undefined basepairs, i.e. AGGTCAnnnTGACCT. The D-box is a subregion in the N-terminal base of the second zinc finger that has an import function in distinguishing the number of nucleotides spacing the half-sites of different hormone response elements. In addition, the D-box harbors a dimerization interface that together with sequences in the LBD provide

surfaces for interaction between the two estrogen receptor molecules at the formation of either ER-homodimers or heterodimers, a prerequisite for high affinity ERE-binding.

Hinge domain.

The D-domain is a short and flexible region that serves as a hinge between the DBD and the LBD. The hinge domain, which is not very well conserved between ERα and ERβ (30%), appears to be important for nuclear translocation and has been reported to contain a nuclear localization signal (Picard et al., 1990). However, this region may also harbor additional properties since, for other NRs, the hinge region has been reported to be of importance for interaction with corepressor proteins (Chen and Evans, 1995).

Ligand binding domain.

The E-domain is the ligand-binding domain and the ERs, α and β, share approximately 55% amino acid identity in this region. The LBD is multifunctional since, in addition to ligand binding, it also mediates receptor dimerization (Kumar and Chambon, 1988) and contains a hormone-dependent activation function (AF-2) (Tora et al., 1989). The three-dimensional structures (3D) of the LBDs of both ERα and ERβ have been determined in the presence of agonists or antagonists (Brzozowski et al., 1997; Pike et al., 1999; Shiau et al., 1998)(Fig. 4). These studies have revealed that the LBDs of ERα and ERβ have a very similar 3D-structure, however, the amino acids lining the ligand-binding cavities of ERα and ERβ differ in two positions. Furthermore, the ligand-binding cavity of ERβ is significantly smaller (approximately 20%) than the ligand-binding cavity of ERα and this may have implications for the selective affinity and pharmacology of ligands. In fact, although the high degree of similarity of the ERα and ERβ 3D-structures suggests that the majority of ligands would bind the two subtypes with similar affinity, ligands that exhibit distinct binding characteristics to the respective subtype have been reported (Barkhem et al., 1998; Kuiper et al., 1997; Sun et al., 1999).

The structures of ER in complex with agonist or antagonist have revealed that, although both types of ligands bind to the same site within the LBD, each ligand induces different receptor conformations. Subsequent to binding of ligand the receptor 3D structure is transformed to adopt an agonist or antagonist conformation depending on the type of ligand bound (Fig. 4). The exact protein conformation of the LBD is made up of 12 α-helices (H1-H12). Once a full agonist binds to the ligand-binding cavity, the H12 is translocated into a conformation sealing the agonist in the cavity. This transformation exposes the AF-2 region on H12 for coactivator protein interactions. When antagonists such as raloxifene or tamoxifen are bound to the ligand-binding cavity, the H12 is positioned along a shallow groove between H3, H4 and H5, with the consequence that the AF-2 region is not accessible for coactivator interaction and the contribution of transcriptional activity from AF-2 is blocked.

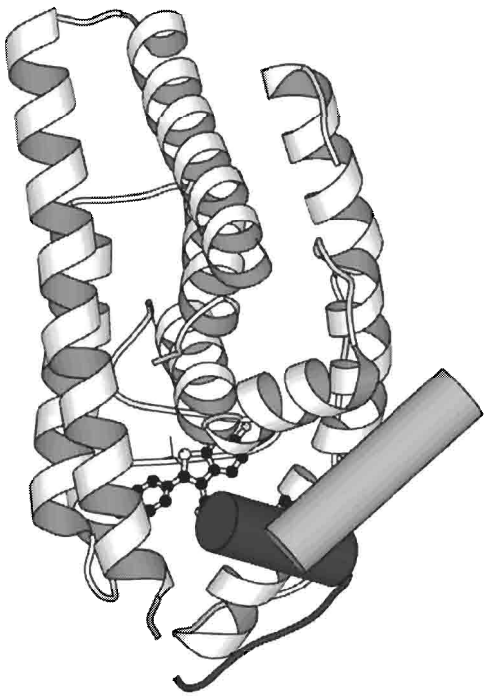


Figure 4. Positioning of helix 12 (H12) in ERα–LBD 3D structure. The black cylinder represents the position of H12 in the agonist-bound ERα whereas the grey cylinder shows the position of H12 in the antagonist structure.

F –domain.

The F-domain in the C-terminal end of the receptor molecule has less than 20% identity between the two ER subtypes and its function remains largely undefined. However, it has been reported that ERα devoid of the F-domain has reduced transcriptional activity in response to estrogen agonists and that antiestrogens were less potent in suppressing E2 stimulated transcription (Montano et al., 1995).

Nuclear Receptor Coregulators.

p160 coactivators.

Coactivators are defined as proteins that interact with NRs and enhance their transcriptional activity. Many coactivators have been identified through their ligand-dependent interaction with NRs in yeast two-hybrid systems. However, the first protein shown to interact with ERα in a ligand-specific fashion was a protein of approximately 160 kD in size, identified by a biochemical approach (Halachmi et al., 1994). In a GST-pulldown assay using ERα-LBD as bait, a 160 kD protein was shown to interact specifically with E2 liganded ERα-LBD and was subsequently cloned and named SRC-1/NCoA-1 (henceforth referred to as SRC-1)(Onate et al., 1995), which has served as a prototypic coactivator for different NRs. SRC-1 is a member of the p160 family of coactivators. So far, two other p160 subtypes have been cloned, TIF-2/GRIP-1/NCoA-2/SRC-2 (henceforth referred to as TIF-2) (Hong et al., 1997; Voegel et al., 1996), and pCIP/ACTR/AIB1/SRC-3 (henceforth referred to as SRC-3) (Anzick et al., 1997; Chen et al., 1997)

Structure-function analysis of the p160s revealed three copies of the signature motif, LXXLL (NR-box) (in the single letter amino acid code where X is any amino acid), present in the central region of all the p160 coactivator subtypes. The NR-boxes were shown to be required for interaction with the AF-2 of NRs (Heery et al., 1997). Subsequent studies revealed LXXLL motifs also in other classes of NR coactivators (Heery et al., 1997; Yuan et al., 1998). Extensive mutagenesis analysis showed that different LXXLL motifs within SRC-1 were selectively required to support functions of

different NRs. The selectivity is proposed to be dependent on the amino acids located adjacent to the LXXLL motifs (McInerney et al., 1998a). Thus, it has been shown that SRC-1 and TIF-2 preferentially interact with ERα via their second NR box motif (Ding et al., 1998; Torchia et al., 1997). Furthermore, SRC-3 has been shown to enhance ERα stimulated gene transcription whereas ERβ- mediated transcription was unaffected (Suen et al., 1998).

The three members of the p160 family show a sequence similarity of approximately 40% and share functional characteristics. Apart from the ligand-dependent interaction with different NRs that results in an increased transcriptional activity, overexpression of the p160 proteins relieve squelching, indicating that they are limiting factors at transcriptional activation of target genes by NRs and their cognate hormone (Voegel et al., 1996). Furthermore, the important role of p160 coactivators in nuclear receptor mediated transcription has been confirmed in loss of function studies using microinjected antibodies against the p160 coactivators. Interestingly, increased cellular content of p160 coactivators may have implications on human carcinogenesis since SRC-3 was shown to be frequently overexpressed in breast tumors (Anzick et al., 1997).

The N-terminal region of the p160 coactivators contains bHLH/PAS (basic helix-loop-helix and Per/Arnt/ Sim homology) domains (Fig. 5), which have a potential role in interactions with other protein factors. The p160s contain two major transactivation domains (AD) of different strength, located in the C-terminal region of the coactivator protein. The stronger activation domain serves as a docking site of the CREB-binding protein (CBP) (Chakravarti et al., 1996; Torchia et al., 1997; Voegel et al., 1998) that possesses HAT activity. However, p160 coactivators appear to have intrinsic HAT activity that also has been mapped to the C-terminus of the proteins. The weaker of the two activation domains, located in the outermost C-terminal region, has recently been shown to interact with an arginine methyltransferase (Chen et al., 1999a) that results in enhancement of transcription, a process that is little understood so far.

Targeted disruption of the SRC-1 gene in mice resulted in a phenotype that was viable and fertile (Xu et al., 1998) but the responses to E2 in estrogen-target tissues, e.g. the uterus and mammary glands, were decreased. In addition, there was an upregulation of TIF-2 in the SRC-1 deficient mice, but not of SRC-3. This indicates that TIF-2 may, at least partially, compensate for the loss of SRC-1. Subsequently, mice have been

generated in which SRC-3 was genetically disrupted. These mice exhibited a distinct phenotype as compared to the SRC-1 null mice, except with respect to mammary gland growth retardation, which was observed also in the SRC-1 knockout mice (Xu et al., 2000). The phenotype of SRC-3 knockout mice included dwarfism and low estrogen production. All together these studies indicate distinct functions of the p160 coactivators.

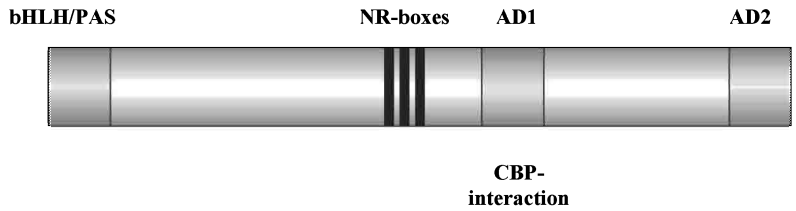


Figure 5. Overview of the structural organization of p160 coactivators.

CBP/p300.

CBP and p300 are highly homologous proteins that serve a more general role as transcriptional coactivators as compared to the p160 coactivators that are mainly restricted to NR signaling. The CBP/p300 proteins interact with the p160 coactivators but may also interact directly with ER through LXXLL motifs located in the N-terminal region of the proteins. In addition, CBP/p300 have been reported to interact with a large variety of transcription factors including CREB, AP1 and NF-κB (Giordano and Avantaggiati, 1999). CBP/p300 may also interact with the basal transcription factors (Swope et al., 1996). Based on its competence for multiple interactions with different types of transcriptional activators, it has been suggested that CBP/p300 may serve as a cointegrator of multiple signal transduction pathways within the nucleus, linking these to the basal transcription machinery.

CBP/p300 has strong HAT activity through a region in the central part of the protein (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), but CBP/p300 also

contain docking sites for other coactivators, such as pCAF, that possess HAT activity. Although CBP and p300 are evolutionarily conserved they appear to play distinct roles in the transcriptional activating process since knock-out of p300 in mice was shown to be lethal, showing that CBP was unable to compensate for the lack of a functional p300 (Yao et al., 1998).

Interestingly, CBP mediated acetylation of lysine residues flanking one of the NR-interacting LXXLL motifs in SRC-3 has been shown to have a negative effect on the interaction between the NR and the LXXLL motif, providing a negative feedback regulation of estrogen-induced transcription (Chen et al., 1999b). This has been supported by chromatin immunoprecipitation studies (ChIP) where p300 and CBP were shown to be recruited to the cathepsin D promoter at different time points and that association of CBP with the promoter correlated with disassembly of the SRC-3-promoter complex (Shang et al., 2000).

TRAP/DRIP complex.

The TRAP/DRIP complex is a large composite coactivator composed of several subunits that was identified due to its interaction with TR (Fondell et al., 1996). In addition, a similar protein complex interacting with VDR has been isolated (Rachez et al., 1998). The TRAP/DRIP complex does not possess HAT activity. The complex has been shown to contain a factor, TRAP 220, that via an LXXLL motif is responsible for its interaction with agonist-liganded NRs (Treuter et al., 1999). Interestingly, ERβ recruits the TRAP 220 protein factor more effectively than ERα (Warnmark et al., 2001). Several subunits of the TRAP/DRIP complex have been reported to interact with the C-terminal domain of RNA polymerase II (Jiang et al., 1998). Thus, the TRAP/DRIP complex may constitute a bridge between NRs and the basal transcription machinery, however, its relation to the putative complex that contains CBP and p160 proteins is presently unknown. A potential functional model involves a sequential mode of action, whereby the putative complex that includes p160 and CBP, remodels the chromatin structure, providing access of the TRAP/DRIP complex to the promoter.

p68.

p68 is a recently isolated ERα specific coactivator that previously has been shown to possess RNA-helicase activity. However, deletion experiments have proven that the helicase activity does not appear to be essential for its coactivating function. Interestingly, p68 interacts specifically with the A/B domain of ERα whereas it does not interact with ERα LBD or ERβ. Thus, p68 appears to be an AF-1 specific coactivator for ERα (Endoh et al., 1999). Phosphorylation of a specific serine residue in the A/B domain of ERα was shown to increase the transcriptional activity of p68 (Endoh et al., 1999).

Corepressors.

The corepressors N-CoR and SMRT interact with unliganded type II NRs that are constitutively bound to DNA. This results in repression of the basal transcriptional activity. Furthermore, N-CoR and SMRT have been shown to interact also with antagonist bound steroid receptor. For instance, they appear to be important mediators of the inhibitory effect of estrogen receptor antagonists, e.g. tamoxifen (Lavinsky et al., 1998; Smith et al., 1997). Several lines of evidence suggest that N-CoR and SMRT function as repressors of transcription by recruiting a complex of proteins that possesses histone deacetylase activity (HDAC) (Heinzel et al., 1997; Nagy et al., 1997). Conversely to the action of the coactivator complex, recruitment of the corepressors results in loss of acetyl groups on histones, reestablishing the dense chromatin structure that leads to restricted access of transcription factors to the promoter and repression of transcription. Also corepressors have been shown to contain conserved sequences responsible for NR interaction. These have been referred to as CoRNR boxes and consist of the signature motif LXXI/HIXXXI/L (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). Surprisingly, mutations of amino acid residues in the AF-2 of the NR that directly participate in the NR-coactivator interaction have been shown to abrogate also NR-corepressor interactions. This indicates that the coactivator- and corepressor binding sites of NR, at least in part, overlap with each other (Nagy et al., 1999). Interestingly, tamoxifen resistence in breast cancer cells has been shown to

correlate with reduced levels of corepressors (Lavinsky et al., 1998). In addition, microinjection of antibodies against NCoR converted tamoxifen into an agonist in MCF-7 breast cancer cells. Taken together, these data suggest that a reduced expression level of corepressors is a potential mechanistic explanation for the acquired resistance to tamoxifen in breast cancer patients, a notion that remains to be confirmed.

Another protein, REA (repressor of estrogen receptor activity) has been shown to selectively repress ER activity, suppressing the effect of estrogen agonists and increasing the antagonistic potency of antiestrogens (Delage-Mourroux et al., 2000; Montano et al., 1999). In addition, the orphan nuclear receptors SHP (short heterodimer partner) and DAX-1 (DSS-AHC critical region on the X-chromosome gene 1) have been shown to suppress ER mediated transcriptional activity (Johansson et al., 2000; Johansson et al., 1999; Zhang et al., 2000).

Transactivation.

Activation function 1 (AF-1).

The ER transactivation functions are believed to work by binding coactivators and bringing them to the promoter. The AF-1 located in the N-terminal A/B domain is not well understood. Antiestrogens such as tamoxifen may have partial agonist activity in a certain cell- and promoter context that relies on the AF-1 (Metzger et al., 1992; Tzukerman et al., 1994). A progressive deletion analysis combined with internal deletions of the A/B domain of ERα revealed that different regions within the A/B-domain were required for transcriptional agonism induced by estrogen versus tamoxifen (McInerney and Katzenellenbogen, 1996). Tamoxifen, which shows agonistic activity with ERα, on an ERE-promoter-reporter gene, exhibits no agonistic activity with ERβ (Barkhem et al., 1998). The agonistic activity of tamoxifen was restored with a chimeric ERβ that contained the A/B domain of ERα (McInerney et al., 1998b). This indicates that the AF-1 region of ERβ is considerably weaker than the AF-1 of ERα, at least with respect to ERE-based promoters. In addition, it has been shown that the isolated A/B domain of ERβ, in contrast to the AF-1 of ERα, lacks autonomous ligand-independent transcriptional activity (Cowley et al., 1997; Delaunay et al., 2000).

However, AF-1 mediated transcriptional activity of ERβ on an ERE-based reporter construct has been observed when the p160 protein SRC-1 was overexpressed (Tremblay et al., 1999). Furthermore, this effect was dependent on phosphorylation of critical serine residues in the A/B domain of ERβ.

Also ERα has been reported to interact with p160 coactivators via its A/B domain. Webb et al. showed that overexpression of the p160 protein GRIP-1 (mouse ortholog of TIF-2) increased the transcriptional activity of AF-1 in ERα (Webb et al., 1998). Moreover, the AF-1 was shown to interact with sequences near the C-terminal end of the p160 coactivator in a region devoid of NR-boxes. Recently, a potential α-helical structure within the AF-1 was identified and shown to mediate synergism between SRC-1 and ERα (Metivier et al., 2001). Hence, p160 induced AF-1 activity represents a potential mechanistic explanation for the partial agonism displayed by AF-2 antagonists such as tamoxifen. However, the recent finding of the coactivator, p68, provides an alternative mechanism to explain AF-1 activity. Consistent with its function as an AF-1 specific coactivator, p68 enhanced the partial agonistic activity of ERα in response to tamoxifen, while the activity of the pure ER antagonist ICI 164,384 was not affected (Endoh et al., 1999). The activity of corepressors may also be regulated via the AF-1, but through a mechanism that does not involve direct binding of the corepressor to AF-1. The corepressor SMRT has been shown to attenuate basal transcriptional activity as well as tamoxifen agonist activity, while estrogen-dependent transcription was unaffected (Smith et al., 1997). These observations indicate that the corepressor interacted in a repressive fashion with unliganded or tamoxifen bound ER, but not with estrogen bound ER. In a recent report, Lavinsky et al., showed that the interaction between ER and the corepressor was destabilized by EGF-1 treatment and that a specific serine residue in the A/B domain was critical for the destabilization (Lavinsky et al., 1998). Taken together, these data indicate that although corepressors have not been shown to interact directly with the AF-1, phosphorylation in this region may lead to a displacement of the corepressor from the ER.

Activation function 2 (AF-2).

A significant amount of data has emerged as to how the ligand-dependent AF-2 transactivates target genes. Ligand-dependent activation of transcription by the ERs is mediated by interactions of coactivators with the AF-2 domain. The core region of the AF-2 resides in H12 of the LBD of the ERs and has been studied in great detail through mutational analysis but also lately by crystallographic studies (Brzozowski et al., 1997; Shiau et al., 1998). The 3D structure of ERα bound to the estrogen agonist diethylstilbestrol (DES) in complex with a nuclear receptor-box (NR-box) peptide that contains the specific signature motif (LXXLL) has been determined (Shiau et al., 1998). The motif was derived from the second NR-box of the coactivator GRIP-1. As described above, agonist binding to the ligand-binding cavity within the LBD of ER results in conformational changes of the LBD whereby H12 is positioned so that an interaction surface for coactivators is provided. The 3D structure of diethylstilbestrol-bound ERα in complex with the NR-box peptide revealed that the peptide binds to the hydrophobic groove formed between H3, H4, H5, and H12 on the surface of the LBD. In contrast, when ER is complexed with antagonist, the antagonist side-chain prevents H12 to adopt its agonist position. Instead, H12 adopts a position at the AF-2 surface, which resembles the position of coactivator peptide bound to the agonist-induced structure of ER. Thus, antagonists prevent coactivator binding both by disrupting the structure of the AF-2 surface but also through autoinhibition mediated by H12.

Synergism between AF-1 and AF-2.

The transcriptional activities of both AF-1 and AF-2 of the ERα and ERβ are dependent on recruitment of cofactors. Furthermore, in most promoter contexts, a functional synergism between the weaker AF-1 located in the N-terminal region of the receptor, and the stronger hormone inducible AF-2 in the LBD is required to give full transcriptional response of an ER agonist (Kraus et al., 1995). The basis of the functional synergism between AF-1 and AF-2 is not understood, however, recent data provide some mechanistic insights. For instance, the p160 coactivator TIF-2 may interact with the isolated AF-1 and AF-2 domains of ERα simultaneously, using distinct interaction surfaces. In addition, this simultaneous interaction was demonstrated to

result in strong transcriptional synergism (Benecke et al., 2000). The data indicated that TIF-2 may act as a bridging factor between the N-terminally located AF-1 and the AF-2 in the C-terminus, leading to increased transcriptional activity. Other investigators have demonstrated a similar role for CBP, mediating transcriptional synergism between AF-1 and AF-2 of either ERα or ERβ (Kobayashi et al., 2000). In addition, the ERα specific AF-1 coactivator p68 (Endoh et al., 1999) was shown to mediate synergism between AF-1 and AF-2 of ERα (Watanabe et al., 2001). However, also the presence of TIF-2 and the recently discovered SRA (steroid receptor RNA activator)(Lanz et al., 1999), which is an unusual coactivator since it appears to consist of an RNA molecule, was shown to be required to obtain maximal ligand-induced transcriptional activity.

Selective estrogen receptor modulators.

Estrogen replacement therapy is effective against osteoporosis and perhaps also in the prevention of cardiovascular disease (CVD). It also alleviates the vasomotor flushes that are associated with the menopause. However, unopposed estrogen replacement therapy increases the incidence of endometrial cancer (van Leeuwen and Rookus, 1989; Ziel and Finkle, 1975). In addition, vaginal bleedings may persist after the menopause in women receiving estrogen replacement therapy. Combined therapy with progestins eliminates the risk for endometrial cancer but may increase the risk for breast cancer (Key and Pike, 1988; WHI, 2002). Thus, estrogen-replacement therapy produces side effects in breast and uterus that limit compliance.

Selective estrogen receptor modulators (SERMs) (Fig. 6) are a class of compounds that bind the ER with high affinity and exhibit estrogen agonism in some tissues but block ER action in others. The concept of SERMs is derived from the observation that tamoxifen, used as an adjuvant therapy for the treatment of breast cancer (GROUP, 1988), showed mixed tissue selectivity with respect to estrogen agonism/antagonism. Tamoxifen therapy of postmenopausal women with breast cancer has, in addition to its antiestrogenic effect in breast tissue, estrogen-like effects on bone mineral density (Love et al., 1992), and lipoprotein levels (Love et al., 1990). Unfortunately, tamoxifen also possesses estrogen-like activity in the uterus, where it

stimulates proliferation of the uterine lining and increases the risk of endometrial cancer (Wolf and Jordan, 1992).

Raloxifene is a second generation SERM, available for prevention of osteoporosis, which has been shown to possess more attractive tissue selectivity. Raloxifene is devoid of estrogen agonism in uterine tissue, while it exhibits the beneficial effects of tamoxifen in bone, breast and on serum lipoprotein profile (Black et al., 1994; Fuchs-Young et al., 1995). In addition, raloxifene seems to show estrogen-like effects on the vasculature, promoting vasodilatation (Figtree et al., 1999). However, still raloxifene is not a perfect drug; like tamoxifen it aggravates the problem of vasomotor flushes at menopause (Cummings et al., 1999). Raloxifene also increases the incidence of thromboembolic disease. The magnitude of the increased risk is comparable of the increased risk observed with tamoxifen or estrogen replacement therapy, suggesting similar mechanisms of action. Presently, several different SERMs are under clinical development. However, recently two different SERMs, levormeloxifene and idoxifene, were withdrawn from clinical development due to side effects such as uterine prolapse and urinary incontinence. Currently available SERMs cannot be used to treat the vasomotor disturbances such as hot flushes that are associated with menopause. Thus, intense research to develop compounds that avoid these problems is currently ongoing in the pharmaceutical industry.

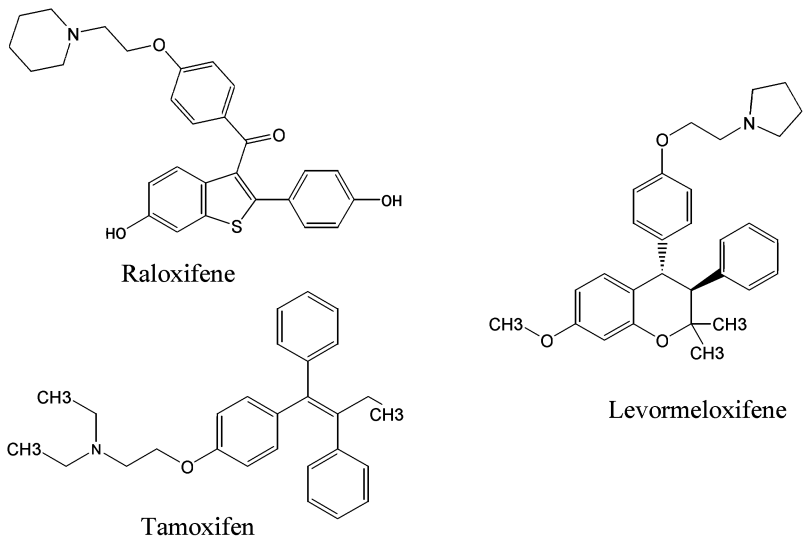


Figure. 6. Molecular structures of SERMs

Molecular determinants of estrogen receptor transcriptional activity.

The molecular mechanisms that determine the transcriptional activity of ER regulated genes are not fully understood. However, during the last decade a significant knowledge on this matter has emerged. The discovery that estrogen agonists and antagonists induce different conformations of the receptor has provided a major leap in our understanding of the processes that control NR regulated genes. Initially, partial protease digestions of agonist- or antagonist-liganded ER revealed different patterns of the cleaved protein, providing the first evidence of ligand-specific conformational changes of the receptor structure (Beekman et al., 1993). The determination of the 3D structures for agonist and antagonist bound ER provided further information, clearly showing H12 acted as a molecular switch, adopting a position that permits the AF-2 of the receptor to interact with associated protein factors in the agonist-bound conformation, whereas the antagonist structure did not allow this type of interactions (Brzozowski et al., 1997; Pike et al., 1999; Shiau et al., 1998).

Another major step in understanding NR function was the discovery of receptor interacting proteins, coactivators and corepressors that modulate the transcriptional activity of NRs on target genes. These findings provided models to explain the tissue selective activity of SERMs, displaying estrogen agonism in one tissue but antagonism in another. Coactivators have been shown to interact with AF-1 and enhance its transcriptional activity also when ER is complexed with a SERM that blocks the function of AF-2 (Endoh et al., 1999; Smith et al., 1997; Webb et al., 1998). In addition, the expression level of corepressors has been shown to influence the agonist/antagonist activity of tamoxifen (Lavinsky et al., 1998). Furthermore, different SERMs appear to induce distinct structural alterations of the ER conformation. This is supported by the identification of a peptide that interacts specifically with tamoxifen-liganded ER, but not with ER in complex with other SERMs (Norris et al., 1999). In addition, a coactivator that interacts with tamoxifen-bound ER but not with ER complexed with estrogen has been described. This protein, L7/SPA (switch protein for antagonists), enhanced the partial agonism of tamoxifen but had no effect on estrogen-mediated transcription (Jackson et al., 1997). Thus, tissue selective expression of various cofactors may explain the mixed agonism/antagonism of SERMs. In addition, subtle structural differences of the ER conformation induced by different SERMs may explain

differences in the agonist/antagonist profile within the SERM family, e.g. the uterothrophic properties of tamoxifen that raloxifene is lacking. Interestingly, a recent study showed that tamoxifen and raloxifene both recruited corepressors to a subset of target gene promoters in mammary cells whereas only raloxifene induced recruitment of corepressors to these promoters in endometrial cells. Furthermore, instead of recruiting corepressors, tamoxifen, but not raloxifene, induced the recruitment of coactivators to the target promoters (Shang and Brown, 2002). This supports the notion that cell type- and promoter-specific recruitment of cofactors determines the cellular response to SERMs.

The discovery of ERβ provided another possible explanation for the tissue-selective activity of different ER ligands (Kuiper et al., 1996). ERβ exhibits a distinct tissue distribution (Kuiper et al., 1997; Taylor and Al-Azzawi, 2000) and may explain estrogen responses in tissues where ERα previously has not been detected. Furthermore, ER subtype selective ligands have been reported (Barkhem et al., 1998; Kuiper et al., 1997; Sun et al., 1999). In addition, the respective ER subtype may as well selectively regulate target genes, which is exemplified by the reverse effect of estrogen via ERβ on the AP1 site of the collagenase promoter (Paech, 1997) and the ERβ specific activation of the iNOS gene in vascular smooth muscle cells (Zhu et al., 2002). Interestingly, the recent report of a human ERβ variant, extended in the N-terminus, provides another potential candidate for mediation of tissue selective effects of SERMs (Wilkinson et al., 2002). This variant of ERβ, in contrast to the shorter forms of ERβ, displayed partial agonism on an ERE-reporter in response to tamoxifen and raloxifene.

Furthermore, a novel mechanism to modulate NR-mediated transcriptional activity was recently proposed. Similar to the ligand-induced conformational changes of the ER structure, the response element has been shown to allosterically modulate the ER conformation (Klinge et al., 2001; Lefstin and Yamamoto, 1998; Wood et al., 1998). Different types of EREs have been shown to modulate ER interaction with p160 coactivators (Hall et al., 2002; Wood et al., 2001), which is likely to affect the transcriptional activity ER-responsive genes.

Thus, the ER mediated modulation of the transcriptional activity of target genes is multifaceted and may be influenced by a variety of parameters. The nature of the ligand as well as the tissue selective expression of ER subtype and cofactors, together with the

type of response element may all modulate the overall transcriptional activity of an ER regulated target gene.

Different modes of transcriptional regulation.

In the absence of ligand the ERs are thought to be held in the nucleus in a complex with heat shock proteins (Baulieu et al., 1990). Upon ligand binding the receptors dissociate from the complex and bind to DNA as homo- or heterodimers (Kumar and Chambon, 1988; Pettersson et al., 1997). The DNA-binding domain enables the receptor to interact with its specific DNA target, the ERE that is usually located in the regulatory flanking sequence of target genes. This is considered to be the classical variant of ER modulation of target genes but lately several alternative regulatory modes have been described for ERα and ERβ.

By preventing interaction of the transcription factor NFκB with its response element, estrogen blocks gene expression of the cytokine IL-6. It has been reported that the repression of IL-6 gene expression was due to protein:protein interaction between ERα and the subunit c-rel of the NFκB complex (Galien and Garcia, 1997).

Another mode of gene regulation that has been proposed for the ERs is activation of gene expression by indirect binding of the receptor to the target promoter. Hence, the ER is tethered to a transcription factor complex that contacts the DNA. Both ERα and ERβ can act on an AP1 site to stimulate gene expression in the presence of antiestrogens such as tamoxifen or raloxifene. E2 stimulated transcription in the presence of ERα while in the presence of ERβ, E2 acted as an antagonist, inhibiting the activity of both tamoxifen and raloxifene (Kushner et al., 2000; Paech, 1997; Webb et al., 1995; Webb et al., 1999). A similar tethering mechanism has been suggested for the ER: Sp1 complex that interacts with the GC-rich Sp1 motif (Batistuzzo de Medeiros, 1997; Porter, 1997). In a study by Zou et al., ERβ activated an RARα1 promoter-reporter construct presumably by the formation of an ER: Sp1 complex (Zou et al., 1999). In the presence of antagonists such as tamoxifen and ICI 164,384, reporter gene expression was induced. This effect was blocked in the presence of E2, which resembles the effect of ERβ on AP1.

Furthermore, ERα has been shown to mediate the stimulatory effect of estrogen on the cyclin D1 promoter whereas ERβ had a repressive effect in response to estrogen (Liu et al., 2002). However, both ERα and ERβ stimulated cyclin D1 gene transcription in response to antiestrogens. These effects were traced to a cAMP response element (CRE) in the cyclin D1 promoter (Liu et al., 2002).

Also the quinone reductase gene, which is involved in detoxification of by-products of metabolic oxidation, is stimulated by antiestrogens via an electrophilic/antioxidant response element (EpRE/ARE) while E2 blocked this effect (Montano, 1997). This may, at least in part, explain the proposed antioxidant effect of the antiestrogen tamoxifen. Interestingly, ERβ was more effective than ERα in stimulating the quinone reductase gene (Montano et al., 1998).

Ligand-independent activation of estrogen receptor.

Steroid independent activation of steroid hormone receptors was first discovered with the chicken PR (Denner et al., 1990). To date, numerous different agents in addition to its natural or synthetic hormones have been shown to activate ER (Aronica and Katzenellenbogen, 1993; Bunone et al., 1996; Kato et al., 1995; Katzenellenbogen and Norman, 1990; Trowbridge et al., 1997; Zwijsen et al., 1997). ER has been demonstrated to be a phosphorylation target *in vitro* for mitogen activated protein kinase (MAPK) (Kato et al., 1995) and polypeptide growth factors that activate the MAPK pathway through membrane receptors have been shown to stimulate ER dependent transcription (Bunone et al., 1996; Kato et al., 1995). The AF-1 domain appears to be required for activation by EGF-1 and IGF-1, since point mutation of a single phosphorylation site within this domain inhibits the ability of these growth factors to activate ERα (Bunone et al., 1996; Kato et al., 1995). The finding that EGF-1 was able to mimic the effect of estrogen in the mouse uterus, an effect that was blocked by the pure antiestrogen ICI 164,384, supports the physiological relevance of ligand-independent activation (Nelson et al., 1991). In addition, ERα deficient mice were unresponsive to the uterothrophic action of EGF-1 (Curtis et al., 1996).

Recent data indicate that the process of ligand-independent activation of ER is complex. Activation of mouse ERα by insulin occurred independently of the AF-1

region in one cell type but not in another (Patrone et al., 1998). Moreover, ERα was activated by EGF-1 in endothelial cells through a process that did not involve AF-1 and MAPK (Karas et al., 1998). In addition, agents that increase intracellular cAMP levels have been reported to stimulate ligand-independent gene activation of ER responsive genes, an event that was shown to be AF-2 dependent (El-Tanani and Green, 1997). Also, cyclins, which are important regulators of cell cycle progression, may activate unliganded ER. Cyclin D1, frequently found to be overexpressed in breast tumors, has been shown to increase ligand-independent transcription of an ERE regulated reporter gene, presumably by recruiting SRC-1 to the LBD of ERα (Zwijsen et al., 1998). This event did not involve cyclin dependent kinases (CDK). Furthermore, p160 coactivators may integrate ER-signaling and growth factor signaling since a p160 coactivator was shown to be phosphorylated by the MAPK signaling pathway resulting in enhanced ER-mediated transcriptional activity (Lopez et al., 2001). However, the physiological significance of ligand-independent activation of ER is still not understood. It is possible that ligand-independent actions of ER provide a mechanism to allow ER-mediated transcription during conditions where the estrogen concentration is low, e.g. in males and postmenopausal women or at certain time-points during the menstrual cycle.

Non-genomic effects of estrogen.

It is now clear that unliganded ERs are mainly located to the cell nucleus. However, recent data suggest that alternative cellular localization occurs. These involve ERs in the vicinity of the cell membrane as well as in the cytoplasm. It has recently been demonstrated that estrogen rapidly increases intracellular Ca^{2+} and cAMP and activates the MAPK cascade (Aronica et al., 1994; Improta-Brears et al., 1999; Migliaccio et al., 1996). The time course of these events was too rapid to be accounted for by changes in gene expression, suggesting non-genomic actions of estrogen. The activation process has been shown to require ER (Improta-Brears et al., 1999; Migliaccio et al., 1996), possibly localized to the cell membrane. Although, membrane associated ER immunoreactivity has been demonstrated using antibodies that recognize different regions of ER (Pappas et al., 1995; Watson et al., 1999), attempts to isolate and structurally characterize a putative membrane ER have so far failed. However, in a

recent study, expression of ERα or ERβ gave rise to both membrane and nuclear receptor protein (Razandi et al., 1999). Affinity studies revealed near identical estrogen binding characteristics but the membrane ER number was only 2% compared with expressed nuclear ER. However, other studies suggest the existence of different types of estrogen binding sites. Estrogen was shown to rapidly induce neuronal excitability in ERα knockout mice, an effect that was not attenuated by the antiestrogen ICI 164,384, suggesting that the response was not ERβ-mediated (Gu et al., 1999).

Furthermore, ERα has been shown to interact with the p85α regulatory subunits of the PI3 kinase; presumably, this event takes place at the cytoplasmic side of the cell membrane (Hisamoto et al., 2001; Simoncini et al., 2000). This interaction resulted in an increased PI3 kinase activity in vascular endothelial cells followed by protein kinase B/AKT and endothelial nitric oxide synthase (eNOS) activation. This causes vasodilatation and represents one potential mechanism for the vasoprotective effect of estrogens.

Activator Protein 1.

The AP1 transcription factor plays a key role in mediating the transcriptional response to a variety of extracellular stimuli, e.g. growth factors, phorbol esters and UV-radiation. Furthermore, AP1 has been found to be involved in different biological processes such as proliferation and apoptosis (Shaulian and Karin, 2001). The AP1 complex is a dimeric transcription factor composed of members of the jun and fos protein families that interacts with the palindromic binding motif TGA(C/G)TCA in target genes (Angel and Karin, 1991). The prototypic AP1 complex was identified as a heterodimer of c-jun and c-fos, however, additional members of each family have subsequently been identified. In contrast to the fos proteins, which require heterodimerization with jun to bind DNA, the jun proteins are able to form homodimers and interact with the AP1 response element. Furthermore, the jun proteins may form heterodimers with members of the activating transcription factor (ATF) family of transcription factors. These complexes preferentially bind the cAMP response element (CRE)(TGACGTCA). Thus, the combination between different types of AP1 subunits

allows formation of a large number of different homo- or heterodimeric AP1 complexes that show selectivity in their preference for target DNA.

MAPKs are well recognized to modulate the transcriptional activity of the AP1 complex. The MAPKs consist of three families of protein kinases, the extra cellular signal regulated protein kinases (ERK 1, 2), the Jun N-terminal kinases (JNK) and the p38 kinases, all which may transduce extracellular signals from the cell membrane to influence the activity of the AP1 transcription factors. The transcriptional response of the c-fos gene is mediated via the so-called serum response element (SRE) in the promoter region of the gene. The SRE is targeted by a protein complex that consists of the serum response factor and the ternary complex factor, the latter of which is transcriptionally activated through phosphorylation by the MAPK signal transduction pathway. All three groups of MAPKs have been shown to phosphorylate the ternary complex factors, resulting in c-fos expression (Price et al., 1996). The c-jun gene is activated via a CRE binding site in its promoter, which is preferentially targeted by jun/ATF-2 heterodimers. In addition, c-jun is rapidly activated through phosphorylation by JNK. Thus, the c-jun gene is both a target for the MAPK cascade and encodes a protein that mediates MAPK signaling into a transcriptional response.

Fos-Jun family members have been reported to interact with a variety of different proteins, which include both DNA binding proteins and transcriptional coactivators that do not bind DNA directly (Chinenov and Kerppola, 2001). Different types of transcriptional crosstalk between AP1 and steroid receptors have been observed. As commented on above, estrogen signaling has been traced to AP1 response elements (Kushner et al., 2000; Paech, 1997; Webb et al., 1995; Webb et al., 1999). However, crosstalk between GR and AP1 is most extensively studied. In particular, interactions between GR and AP1 have been shown to negatively interfere with the respective activity of the two transcription factors (Jonat et al., 1990; Schule et al., 1990). Furthermore, GR can either stimulate or repress transcription at so-called composite response elements that contain binding sites for both GR and AP1 (Diamond et al., 1990). Interestingly, the composition of the AP1 complex determined the transcriptional response of these promoter elements since c-jun homodimers were shown to stimulate GR mediated transcription whereas c-jun/c-fos heterodimers had a repressive effect (Diamond et al., 1990).

Physiological effects of estrogen.

The female reproductive system.

Estrogens have a broad range of target tissues in the human body (Nilsson and Gustafsson, 2002). Estrogen is required for female pubertal development and affects growth, differentation and function of the female reproductive system. The major target tissues include the mammary gland and the urogenital tract. Both ERα and ERβ are expressed in the mammary glands (Taylor and Al-Azzawi, 2000). The mammary glands of ERα-deficient (ERKO) female mice fail to develop and exhibit a phenotype that is similar to the glands of a newborn female wild type mouse (Bocchinfuso, 1997). No such phenotype was observed in ERβ-deficient (BERKO) female mice (Couse and Korach, 1999). BERKO females exhibited a normal ductal structure of the mammary glands, which was indistinguishable from the structure in wild type mice of the same age. Interestingly, tissue recombination experiments using tissue from the stromal compartment and from the ductal epithelium of wild type and ERKO mice revealed that the presence of ERα was required in the stroma, but not in the epithelium, to induce E2-dependent growth of the ductal epithelium (Cunha et al., 1997). This indicates that E2 induces stromal secretion of paracrine factors that mediate the E2-dependent mitogenic activity on the ductal epithelium in the mammary glands.

Female ERKO mice are infertile with an insensitivity of the uterus to estrogen treatment and have ovaries that contain hemorragic cystic follicles and no corpora lutea (Korach et al., 1996; Pendaries et al., 2002). The serum gonadotropin levels in female ERKO mice are increased although only luteinizing hormone (LH) is substantially elevated (Scully et al., 1997). However, normalization of serum gonadotrophin levels, using a gonadotrophin releasing hormone antagonist, rescues the ovarian phenotype, indicating that the ovarian phenotype of the ERKO mice is entirely caused by the elevated gonadotrophins (Couse et al., 1999). These observations, together with the fact that the serum levels of E2 are tenfold elevated in female ERKO mice, indicate that ERα is an important regulator of the hypothalamic-pituitary-gonadal axis. Also ERβ is important in the female reproductive system. Although estrogen receptor β knockout (BERKO) females are fertile, the number and size of litters are significantly reduced (Krege et al., 1998). Moreover, the ovaries show signs of follicular arrest and

anovulation (Krege et al., 1998). Thus, it appears that ERβ is the most important intrinsic mediator of estrogen signaling in the ovary.

The male urogenital tract.

Recent data indicate that estrogen also has an important role in the male reproductive system. Male patients with defective estrogen production (Morishima et al., 1995) have reduced fertility and male ERKO mice are infertile due to testicular atrophy and disrupted spermatogenesis (Eddy et al., 1996). In contrast to the male ERKO mice, male BERKOs are fertile, suggesting a different role for ERα and ERβ in the male reproductive system (Krege et al., 1998). The bladder and the prostate are tissues in which ERβ is the predominant receptor subtype and BERKO animals show signs of hyperplasia in the bladder and prostate epithelium (Krege et al., 1998; Weihua et al., 2001). These findings may indicate that ERβ plays a role in regulating growth in the male urogenital tract.

Effects of estrogen in bone.

In addition to its role in reproduction and in the urogenital tract, estrogen affects a number of other physiological systems. In the skeleton, estrogens prevent bone-resorption by inhibition of osteoclast function (Jilka et al., 1992). Estrogen replacement therapy conclusively reduces osteoporosis in postmenopausal women (Meema and Meema, 1968; WHI, 2002). The report of a male patient with severe osteoporosis and unclosed epiphyses resulting in continued linear growth due to an inactivating mutation in ERα (Smith et al., 1994) stresses the importance of estrogen also for development and maintenance of the male skeleton. Similar defects in bone have been reported both in male and female patients with aromatase deficiency (Carani et al., 1997). Administration of exogenous estrogen to these patients resulted in epiphyseal closure and increased bone mineral density, while the bone status of the ERα defective patient was unchanged after estrogen treatment (Smith et al., 1994).

Both ERα and ERβ are present in osteoblasts (Arts et al., 1997; Onoe et al., 1997), whereas ERα has been detected in pre-osteoclastic cells but not in mature

osteoclasts (Oreffo et al., 1999). Estrogen is known to downregulate the synthesis of cytokines such as IL-1, IL-6 and TNFα, which are factors promoting bone resorption, mainly by increasing the number of osteoclast precursors in bone marrow (Manolagas, 2000; Pacifici, 1996). Estrogen has also been shown to suppress the recently discovered OPG/RANKL/RANK pathway that mediates the final step in osteoclast differentiation. The receptor activator of NF-κB ligand (RANKL) is expressed on the surface of pre-osteoblastic stromal cells. Contact between these cells and osteoclast precursor cells allows binding of RANKL to its physiological receptor (RANK) expressed on the osteoclast precursor cells, which results in osteoclast differentiation. However, the pre-osteoblastic stromal cells also secret osteoprotegerin (OPG), which is a soluble decoy receptor that binds to RANKL, preventing its binding to RANK. Estrogen has been shown to upregulate the expression of OPG, thus neutralizing the osteoclast differentiation pathway (Hofbauer et al., 2000).

However, it has recently been reported that estrogen may also increase the osteoblast content in the skeleton. Estrogen decreased apoptosis of osteoblasts via a rapid pathway involving either ERα or ERβ as well as activation of the Src/Shc/ERK pathway (Kousteni et al., 2001).

The cardiovascular system.

The incidence of cardiovascular disease (CVD) is significantly higher in men than in women. The incidence of CVD rises in postmenopausal women, whereas observational studies have suggested that postmenopausal women receiving estrogen-replacement therapy may have reduced risk (Grady et al., 1992; Stampfer et al., 1991). Moreover, a young male patient with a disruptive mutation in the ERα gene showed premature artherosclerosis (Sudhir et al., 1997). Thus, estrogen appears to play an important role in the cardiovascular system.

Both ERα and ERβ are expressed in vessels (Iafrati et al., 1997). The E2 mediated production of the potent vasodilator, nitric oxide (NO), has been reported to be abolished in ERα-deficient mice (ERKO) (Pendaries et al., 2002), indicating that ERα has beneficial direct effects on the vasculature. Also ERβ has been shown to have effects on vasodilatation. ERβ-deficient mice (BERKO) develop hypertension as they

age (Zhu et al., 2002). In vascular tissue from wild type mice, estrogen was shown to attenuate endothelial-independent vasoconstriction whereas the opposite effect of estrogens was seen in BERKO mice. A potential mechanism could be ERβ specific stimulation of inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (Zhu et al., 2002). In addition, ERα was shown to decrease the transcriptional activity of an iNOS-promoter-reporter gene in response to estrogen, consistent with the estrogen-enhanced vasoconstriction observed in vascular tissue from BERKO mice.

It has been demonstrated that ERβ is strongly upregulated in mice following arterial injury (Lindner et al., 1998). Furthermore, estrogen has been shown to inhibit proliferation of smooth muscle cells in response to arterial injury, which is thought to be an initial step in the onset of atherosclerosis (Iafrati et al., 1997). This effect was initially attributed to ERβ since estrogen still inhibited smooth muscle cell proliferation after experimental injury in ERKO mice (Iafrati et al., 1997). However, recent studies have revealed residual ERα activity in the ERKO mice initially used (Pendaries et al., 2002). A subsequent study using mice fully null for ERα has confirmed that ERα mediates the protective effects of estrogen against vascular injury (Pare et al., 2002).

Estrogens may also indirectly exert protective effects on the vasculature. The effect of estrogen on liver-specific gene expression results in decreased total serum cholesterol and an improved HDL/LDL cholesterol ratio that is thought to prevent progression of atherosclerosis and CVD (Nabulsi et al., 1993). ERα appears to be the predominant receptor in the liver (Kuiper et al., 1997). In ERKO mice the level of liver-specific ApoE expression is decreased (Srivastava et al., 1997). Furthermore, estrogen upregulates the number of hepatic LDL receptors at the transcriptional level (Croston et al., 1997; Kovanen et al., 1979; Ma et al., 1986). Moreover, the levels of SHBG, thyroxine-binding globulin and cortisol binding globulin were reported to be insensitive to exogenous estrogen treatment in the male ERα defective patient (Smith et al., 1994). These observations indicate an important regulatory role of ERα in hepatic tissue.

Thus, many reports indicate that estrogen has a profound effect on risk factors of cardiovascular disease and that both ERα and ERβ contribute to these effects. However, a beneficial effect on the incidence of CVD in women receiving estrogen replacement therapy has not been confirmed in large randomized clinical trials. The Heart and Estrogen/progestin Replacement Study (HERS study) showed no significant decrease in

the incidence of CVD (Hulley et al., 1998). The HERS study may have limitations since the patients recruited to the trial had a history of CVD (Foody, 1999; Kooistra and Emeis, 1999). However, a second randomized trial on women receiving estrogen/progestins; the Women´s Health Initiative study (WHI, 2002), was stopped early based on health risks, including CVD, which exceeded health benefits. Additional clinical trials are required to clarify the effect of estrogen alone, given to women that have had hysterectomy.

Effects of estrogen in the CNS.

In the CNS estrogen controls the hypothalamic-pituitary-gonadal axis and numerous other effects such as beneficial effects on learning and memory (Birge, 1996; Lamberts et al., 1997). Furthermore, it has been claimed that the risk of developing Alzheimer´s disease is lower among women who receive estrogen-replacement therapy compared to non-users (Fillit, 1994; Lichtman, 1996).

ERα and ERβ are expressed in the brain and their individual distribution in the rat brain has been evaluated through the use of *in situ* hybridization (Osterlund et al., 1998; Shughrue et al., 1997a). The expression of ERβ in brain regions that are associated with learning and memory suggests a role of ERβ in these functions.

Estrogen affects thermoregulation that is manifested through hot flushes as a result of decreased estrogen secretion at menopause. Both ERα and ERβ mRNA have been detected in the region of the hypothalamus involved in thermoregulation. The induction of progesterone receptor (PR) in this region of the brain in response to estrogen was studied in ovariectomized wildtype and ERKO mice (Shughrue et al., 1997b). PR expression was attenuated in ERKO mice but still responded to estrogen treatment. Thus, both ER subtypes regulate gene expression in the thermoregulatory center and may contribute to the relief of vasomotor flushes by estrogen.

Aims of the present study.

The aim of this work was to gain further insights into the mechanisms that control the transcriptional activity of estrogen and antiestrogen regulated genes. The ERα and ERβ are key mediators of these effects although non-ER mediated effects also occur. Thus, estrogen signaling is multifaceted and may involve crosstalk between the ERs and many different signaling pathways. In this thesis different aspects of this topic have been addressed. Thus, the specific aims of this thesis were to:

-establish a cellular system to assess the estrogenic character of SERMs in liver cells.

-establish ERα and ERβ specific reporter cell lines and evaluate whether already existing ligands display subtype selective properties.

- investigate the molecular mechanisms that control the pS2 gene promoter in response to estrogen and the phorbol ester PMA.

Results and discussion.

Non-ER dependent activation of SHBG gene expression (paper I).

Tamoxifen and raloxifene, which are referred to as SERMs are both known to lower the serum level of total cholesterol and alter the HDL/LDL cholesterol ratio in a favorable direction. The beneficial effect on the blood lipid profile has been attributed to their estrogenic character in the liver, where they are thought to modulate expression of liver-specific genes involved in cholesterol homeostasis in a similar direction as estrogen.

In the present study (I) (Barkhem et al., 1997), we used HepG2 cells that lack endogenous ER and HepG2 cells stably transformed to express physiological levels of ERα (HepER3) to assess the effect of tamoxifen and raloxifene. Transient transfection experiments of HepER3 cells with an artificial ERE controlled promoter-reporter revealed that both tamoxifen and raloxifene behaved, in principle, as pure estrogen antagonists in this cell and promoter context. Based on this observation, we chose to include endogenous marker genes in our study on the estrogen agonism of tamoxifen and raloxifene in liver cells. SHBG is a glycoprotein synthesized in the liver, whose expression is stimulated by estrogen and tamoxifen *in vivo* (Anderson, 1974; Bruning et al., 1988). The elevated serum levels of SHBG, induced by estrogen or tamoxifen, have been shown to correlate with an improved blood lipid profile.

We showed that tamoxifen and raloxifene stimulated the expression of endogenous SHBG in HepER3 cells at doses above 1 μM of the respective compound (Barkhem et al., 1997). These data suggested that SHBG was a suitable marker gene to assess the effect of SERMs in liver cells. Interestingly, we were able to conclude that the "high dose" effects of tamoxifen or raloxifene on SHBG expression were mediated independently of ERα because the effect remained in its absence (HepG2 cells) (Fig. 7). A small but significant ER-mediated effect (15% relative to the agonism of the estrogen analogue moxestrol) on SHBG expression in HepER3 cells was observed at lower doses of tamoxifen or raloxifene than 1 μM. Whether this low degree of agonism *in vitro* is responsible for the decrease of serum cholesterol levels in postmenopausal women is not known. However, the non-ER mediated SHBG induction by tamoxifen or raloxifene and the ER-dependent effect on SHBG expression of moxestrol displayed a similar efficacy in the HepER3 cells. It is therefore possible that the *in vivo* effect of

tamoxifen and raloxifene in liver is derived from non-ER dependent agonism. Similar results were obtained using an SHBG promoter-reporter construct, suggesting that the non-ER dependent stimulation of SHBG-gene expression was a transcriptional effect.

Furthermore, we were able to show that the non-ER mediated effect was additive to the effect of moxestrol. This observation is in agreement with the additive effect of tamoxifen in pre-menopausal women, who were reported to show an increase of SHBG serum levels (Bruning et al., 1988), similar to that in our cellular system.

In addition to tamoxifen´s function as an estrogen antagonist, a variety of other actions by tamoxifen have been reported, including an ability to inhibit protein kinase C (PKC) (Gundimeda et al., 1996). The fact that the cytokine IL-1β and the phorbol ester, phorbol 12-myristate 13-acetate (PMA), blocked the non-ER dependent activation of SHBG may indicate that the mechanism of tamoxifen and raloxifene action in the liver cells is due to interference with intracellular signal transduction pathways. One possibility is that the non-ER mediated activation of SHBG gene expression involves inhibition of PKC, because the stimulatory effect of tamoxifen was mimicked by bisindolylmaleimide, a potent inhibitor of PKC (data not shown). PMA is a potent activator of PKC and a recent report indicates that also IL-1β is able to stimulate PKC activity (Lin et al., 2000). A speculative model could be that PKC modulates the activity of a transcriptional repressor. When phosphorylated via the PKC cascade, the repressor blocks SHBG gene expression, while inactivation of PKC by tamoxifen or bisindolylmaleimide leads to release of the repressor and activation of SHBG gene expression.

Another explanation is perhaps that the non-ER dependent effect of tamoxifen and raloxifene is mediated by a nuclear receptor. For instance PXR, which is synthesized in the liver, is activated by a variety of compounds, including tamoxifen, at ligand concentrations above 1μM (Blumberg et al., 1998). However, we have not been able to identify a typical PXR response element in the SHBG promoter region.

Thus, this study demonstrated that tamoxifen and raloxifene were able to stimulate SHBG gene expression in a non-ER dependent fashion, which was additive to the ER mediated effect of estrogen. Furthermore, by the use of IL-1β or PMA it was possible to discriminate between non-ER and ER mediated stimulation of SHBG gene expression.

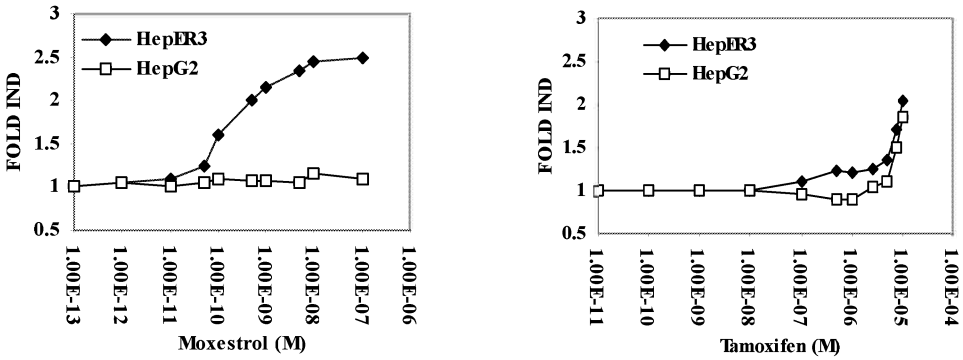


Figure 7. Tamoxifen stimulates SHBG gene expression in a non-ER dependent fashion.

Receptor subtype-selective ER ligands (paper II).

The unexpected discovery of ERβ promises a novel opportunity to develop tissue selective ligands. In paper II of this thesis, we report on the establishment of ERα and ERβ specific ERE-based reporter cell lines (293/hERα and 293/hERβ) and show receptor subtype selective responses to various estrogen agonists and antagonists (Barkhem et al., 1998). We used the ERβ cDNA encoding a protein of 485 amino acids, which was the size of the first ERβ protein reported (Kuiper et al., 1996). The parental cells were 293 human embryonal kidney cells that were chosen since they lack endogenous ER.

The homology between ERα and ERβ in the region of the LBD that is lining the ligand-binding cavity is very high. Thus, many ligands, including E2, exhibit approximately the same potency in terms of transcriptional activation. However, there were ligands that displayed ERα or ERβ selectivity, e.g. the 35-fold ERα selective compound 17α-ethynyl-17β-estradiol or 16β, 17α-epiestriol, which was seven-fold ERβ selective. Another selective compound was the phytoestrogen genistein that previously has been shown to have about 30-fold higher affinity for ERβ than for ERα (Kuiper et al., 1997). This was not completely reflected in our reporter cell lines, since the potency of genistein in the 293/hERβ cells was only about five-fold higher than in the 293/hERα reporter cells. Interestingly, genistein showed receptor selective efficacy, exhibiting partial estrogen agonism via ERβ, whereas ERα mediated a slight superagonism. The presentation of the 3D-structure of ERβ in complex with genistein (Pike et al., 1999) revealed that H12 does not adopt the typical agonist conformation (Brzozowski et al., 1997) but instead is positioned in a similar orientation to that induced by antagonists (Brzozowski et al., 1997; Pike et al., 1999; Shiau et al., 1998). However, there is a 25° difference in the orientation of H12 between the antagonist complex and the ERβ:genistein complex. It is possible that the imperfect antagonist structure adopted by H12 in the ERβ-genistein complex may permit cofactors to force H12 into a more agonist-like position, resulting in partial agonist activity. This is supported by recent observations showing that genistein-bound ERβ binds to NR box peptides with high affinity (Bramlett et al., 2001; Hall et al., 2000). One explanation for the super-agonistic character of genistein via ERα could be that the H12 in the ERα-

genistein complex adopts an orientation similar to that observed when ERα is complexed with full agonists such as E2 or DES (Brzozowski et al., 1997; Shiau et al., 1998). However, an alternative explanation for the difference in agonistic activity of genistein via ERα and ERβ could also be that the reporter cells do not express sufficient levels of ERβ specific coactivators, while ERα specific coactivators are in excess. That may also explain the decreased ERβ selective potency of genistein in the reporter cells as compared to its ERβ selective affinity.

The agonist/antagonist activity of SERMs and the pure ER-antagonist ICI 164, 384 was examined in the 293/hERα and 293/hERβ cells, respectively. Tamoxifen and raloxifene displayed a low degree of agonism in the 293/hERα cells, whereas none of them displayed any agonism in the 293/hERβ cells. All the ligands antagonized the response of E2 with a similar potency in both cell lines, except for raloxifene, which showed 15-fold ERα selective antagonism.

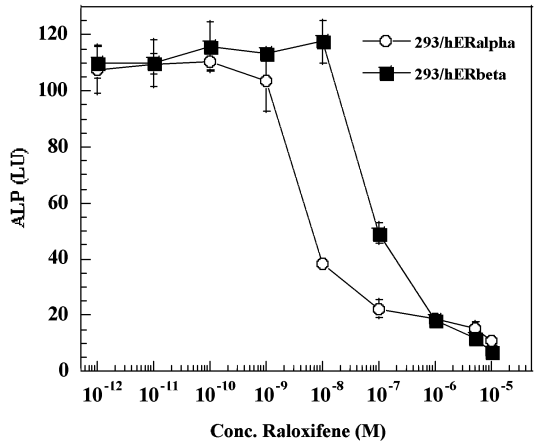


Fig. 8. ERα selective antagonism of raloxifene in E2 stimulated reporter cell-lines.

The different transcriptional activity of ERα and ERβ in response to tamoxifen or raloxifene is probably due to differences in their respective AF-1 domains since both ligands most likely inactivate the ligand dependent AF-2 domain (Brzozowski et al., 1997; Shiau et al., 1998). The partial agonism of tamoxifen has been mapped to a

specific region of the AF-1 domain in ERα (McInerney and Katzenellenbogen, 1996). A plausible explanation for the absence of partial tamoxifen agonism in 293/hERβ reporter cells is that ERβ lacks this particular region. This is supported by the observation that chimeras of ERα and ERβ that contain an AF-1 domain derived from ERα displayed partial tamoxifen agonism (McInerney et al., 1998b).

The antagonism of raloxifene and ICI 164,384 was further analyzed in the reporter cell lines and compared with their binding affinity to ERα and ERβ, respectively. The antagonistic activity of raloxifene and ICI 164,384 in the cells was examined by repeated dose-titration of E2 in the presence of increasing fixed doses of raloxifene and ICI 164,384, respectively. Both compounds were competitive antagonists to E2 in ERα– and ERβ–reporter cells. The ERα selectivity of raloxifene was manifested by a much greater impact on the shift of the EC_{50} value for E2 in the ERα cells than in the ERβ-reporter cells at increasing concentrations of raloxifene. The effect on the EC_{50} values for E2 at increasing concentrations of ICI 164,384 was similar in both cell lines.

The binding characteristics of raloxifene and ICI 164,384 were studied through their respective influence on the binding affinity of [^{3}H] E2 to ERα and ERβ, in the presence of low fixed concentrations of raloxifene or ICI 164,384. Raloxifene was found to be a more potent inhibitor of [^{3}H] E2 binding to ERα, which is in agreement with its ERα selective character in the reporter cells. However, ICI 164,384 showed ERβ selective affinity, which was not reflected in the cell lines. A proposed mechanism of the pure antagonism of ICI 164,384 was that the compound interferes with ERα dimerization function (Fawell et al., 1990). As a consequence of impaired dimerization, the receptor turnover is increased (Dauvois et al., 1992; Pike et al., 2001). This leads to a reduction in the cellular content of ERα and consequently an inhibition of ERα mediated transcription. The lack of congruence between ERβ selective affinity of ICI 164,384 and antagonist potency in the reporter cells may be explained by the assumption that ICI 164,384 impairs the dimerization function of ERβ as well.

In summary, various estrogen agonists and antagonists showed ER subtype selective transcriptional responses in ERα and ERβ reporter cells. The SERMs, tamoxifen and raloxifene, displayed agonistic activity mediated via ERα but not via

ERβ. We conclude that there are good reasons to believe that it will be possible to develop novel ligands with increased ER subtype selectivity.

Complex regulation of the pS2 gene (paper III).

In the present study, we report on the complex regulation of the pS2 gene in response to estrogen or the phorbol ester PMA, in the context of HepG2 cells. Both estrogen and PMA stimulated pS2 gene expression in the presence of ERα, whereas PMA was also able to induce pS2 expression in the absence of ERα. However, in the non-ER expressing cells the PMA effect was ten-fold reduced, suggesting that ERα potentiated the effect of PMA on pS2 gene expression. Estrogen stimulation of the pS2 gene has previously been shown to be mediated via an imperfect ERE in the pS2 promoter (Berry et al., 1989). We found that an inhibitor of the MAPK cascade was able to block estrogen stimulation of the pS2 gene independently of phosphorylation of serine 118 in ERα. It has previously been demonstrated that both ERα and a c-fos related protein were present in two multiprotein complexes associated with the pS2 promoter segment that spans the ERE (Schuh and Mueller, 1993). The fact that the MAPK cascade may target AP1 response elements on DNA, encouraged us to examine the function of an AP1 motif located in proximity to the ERE in the pS2 promoter. Thus, we decided to dissect the contribution of the ERE and the adjacent AP1 response element, respectively, in mediating the response to estrogen or PMA. These studies were performed using pS2 promoter constructs in which the ERE and/or the AP1 response elements had been mutated. Mutation of the ERE caused only a modest reduction in the response to PMA. However absence of the AP1 motif was deleterious to stimulation of pS2 gene expression by PMA.

Interestingly, both the ERE and the AP1 response elements were required to mediate the effect of estrogen, suggesting that the AP1 element plays a dominant role in the regulation of pS2 gene expression in HepG2 cells. We next showed that ERα was able to interact not only with the ERE but also somehow with the AP1 motif in the context of the pS2 promoter. This was demonstrated with a chimera of the transcriptional activator protein VP-16 and ERα, which to some extent stimulated gene expression from the ERE mutated construct, i.e. via the AP1 motif, whereas VP16 alone did not have any effect. Thus, ERα appears to bring the fusion protein to the pS2

promoter despite the absence of a functional ERE, possibly by interacting with proteins at the AP1 motif. Furthermore, cotransfection of ERα devoid of its A/B domain demonstrated that this construct was able to stimulate the pS2 promoter in response to estrogen but that the stimulatory effect was dependent on an intact AP1 motif.

The transcriptional rate of complex natural promoters that are controlled by NRs depends on synergistic interactions between the nuclear receptor and transcription factors binding to specific sequences adjacent to the hormone responsive element (Chuang et al., 1997; Palomino et al., 1998). With regard to the pS2 promoter, a potential explanation could be that the LBD domain of ERα is able to interact directly or indirectly with factors at the AP1 response element that results in a significant potentiation of the transcriptional response. There are conflicting data reported whether LBD of ERα interacts directly with different components of the AP1 complex (Teyssier et al., 2001; Webb et al., 1995). However, the p160 coactivator SRC-1 has been shown to interact, via its C-terminal subregion, with both c-jun and c-fos (Lee et al., 1998). Thus, we decided to investigate whether p160 proteins, e.g. SRC-1, could be involved in the productive crosstalk between the ERE and AP1 response elements at estrogen induction of the pS2 promoter. We asked whether the presence of an intact AP1 motif in the pS2 promoter could influence the interaction between ERα and the NR-box of p160 coactivators. An LXXLL-peptide was overexpressed together with intact or mutated pS2 promoter-reporter constructs. We observed that a larger amount of the LXXLL-peptide expression vector was required to disrupt ERα mediated transcriptional activity on the pS2 promoter in the presence of an intact AP1 motif. The ERE mutated pS2 promoter construct showed a minimal activity in response to estrogen and its basal activity was not influenced by overexpression of the LXXLL peptide, indicating that the LXXLL peptide did not interfere with the proteins at the AP1 complex. This suggests that, in the intact pS2 promoter, the presence of the AP1 response element stabilized LXXLL interactions at the ERE. A potential mechanistic explanation is that the interaction between the AF-2 of ERE-bound ERα and the LXXLL motif of a coactivator is stabilized by an additional interaction between the coactivator and the AP1 complex.

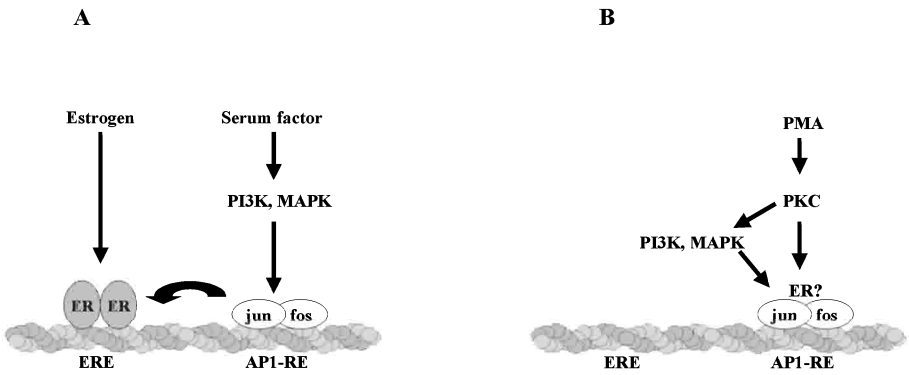


Figure 9. Models for estrogen (A) or PMA (B) activation of the pS2 promoter.

In conclusion, the data presented indicate that ERα plays a crucial role in mediating the effect, not only of estrogen, but also of PMA. Furthermore, the AP1 motif in the pS2 promoter is an essential target on DNA through which various signals converge to modulate pS2 gene expression in the HepG2 cells. Estrogen stimulation of the pS2 promoter required both the ERE and the AP1 motif, indicating a crosstalk between factors at these motifs. We propose that estrogen induction is a result of synergism between the classical ER/ERE pathway and the MAPK pathway that converge on the AP1 motif. In contrast, PMA stimulated the pS2 promoter, via the PKC signal transduction pathway, mainly through the AP1 response element while the ERE played a minor role.

Transcriptional synergism on the pS2 gene promoter by p160 coactivators (paper IV).

The transcriptional activity of the pS2 gene in HepG2 cells is governed by a synergism between an ERE and a nearby AP1 response element in the 5′-flanking promoter region of the gene (paper III)(Barkhem et al., 2002). The present study was undertaken to further characterize the productive crosstalk between the ERE and AP1 response elements of the pS2 promoter and to investigate the effect of p160 coactivators in relation to these promoter sites.

It was found that deletion of the sequence between the ERE and the AP1 response element modulated the transcriptional response of the pS2 gene, suggesting an interaction between factors at the respective site. Our results supported a model whereby a decreased distance between the ERE and the AP1 motif facilitated an interaction between proteins at the respective site, resulting in increased transcription, whereas changing the relative phase position of the response elements on the DNA helix disturbed the interaction and hampered transcription.

The estrogen-induced response of the pS2 promoter was potentiated in a dose dependent fashion when full-length SRC-1 was expressed together with intact or mutated pS2 promoter. The SRC-1-induced potentiation of the ERE or AP1 mutated pS2 promoter variants was, however, substantially less pronounced than the potentiation of the intact promoter. Furthermore, SRC-1 had a minimal effect on the pS2 promoter mutated in both the ERE and the AP1 motif. Thus, overexpression of SRC-1 together with the intact pS2 promoter resulted in transcriptional synergism mediated via the ERE and the AP1 response element. Also the related but distinct p160 coactivator, TIF-2, displayed a strong synergism on the pS2 promoter that was mediated via the ERE and AP1 response elements. In fact, TIF-2 was a more potent activator of pS2 promoter transcription as compared to SRC-1. Overexpression of TIF-2 resulted in approximately 3-fold higher estrogen-induced reporter gene expression from the intact pS2 promoter as compared to the estrogen-induced reporter gene activity seen when an equal amount of SRC-1 expression vector was cotransfected.

A closer examination of the effects evoked by SRC-1 and TIF-2 revealed differences in their dependence of the pS2 promoter context to stimulate transcription via the ERE of the pS2 promoter. The ability of the respective coactivator to potentiate estrogen-induced reporter gene expression in the presence or absence of an intact AP1

response element in the pS2 promoter was therefore examined. Interestingly, SRC-1 showed a much greater dependence on the presence of an intact AP1 motif than TIF-2 in their potentiation of pS2 promoter activity via the ERE. The reason for this might be that TIF-2 interacts with ERα with a stronger affinity than SRC-1. This was supported by a recent study which showed that GRIP-1, the mouse ortholog of TIF-2, interacted with higher affinity, than SRC-1, with ERα bound to the isolated ERE derived from the pS2 promoter (Hall et al., 2002). Hence, TIF-2 might be less dependent on stabilizing interactions with nearby factors at the AP1 response element as compared to SRC-1.

SRC-1 has in addition to its well-known capacity to interact via its NR-box motifs with ERα also the competence to interact via its C-terminal end with the AP1 complex (Lee et al., 1998). Hence, SRC-1 constitutes a potential bridging factor between the ERE and the AP1 response element, interacting via its NR-boxes with the AF-2 of ERα bound to the ERE and via its C-terminal domain with the AP1 complex. To test this concept the C-terminal fragment of SRC-1 (SRC1101-1441) that has been reported to interact with either c-jun or c-fos in GST-pull down experiments (Lee et al., 1998) was overexpressed together with the pS2 promoter-reporter constructs. Interestingly, the intact pS2 promoter was more sensitive to overexpression of the SRC1101-1441 fragment than the AP1 mutated promoter. This is possibly due to binding of the SRC1101-1441 fragment to the AP1 complex, preventing full length SRC-1 to act as a bridging factor between the ERE and the AP1 motif.

Also the type of ERE in the pS2 promoter influenced the degree of potentiation evoked by SRC-1. The pS2 promoter in which the natural ERE was substituted for by a high affinity consensus ERE (EREvit), showed an equal relative potentiation in response to SRC-1 irrespective of the presence or absence of an intact AP1 response element in the pS2 promoter. This contrasts to the influence of the AP1 motif in the wild type pS2 promoter, which showed significantly decreased relative SRC-1-dependent potentiation in the absence of a functional AP1 motif. One potential explanation could be that allosteric modulation of the ER conformation by the respective ERE influences the interaction with coactivator proteins. Interestingly, it was recently reported (Hall et al., 2002) that ERα bound to the isolated pS2 ERE had a much lower affinity for SRC-1 as compared to ERα bound to the EREvit. It seems plausible that ERα bound to the natural ERE of the pS2 promoter recruits SRC-1 less

effectively and therefore relies on additional stabilization of the p160 protein, e.g. via the AP1 motif.

Very few estrogen responsive genes contain consensus EREs. Instead the majority harbors imperfect EREs (Berry et al., 1989; Richard and Zingg, 1990) that bind ER with low affinity. These may also induce a receptor conformation that has a reduced affinity for p160 coactivators (Hall et al., 2002; Wood et al., 2001). One potential reason for this scenario could be that stabilization of the ER-coactivator interaction by adjacent transcription factors provides a mechanism that allows a more exact tuning of gene regulation. This would permit additional signal transduction pathways that converge on other specific sequences in the promoter to influence the transcriptional activity mediated through the hormone response element.

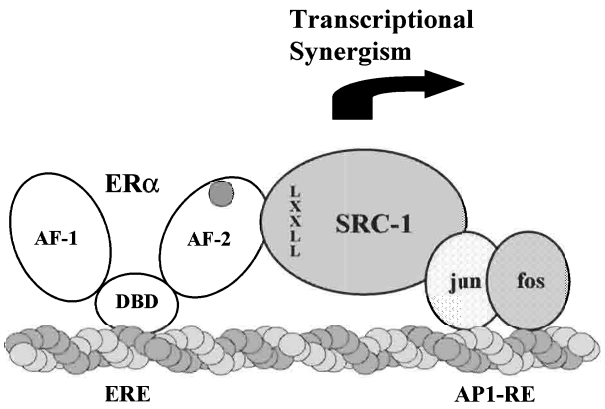


Figure 10. Model of SRC-1 mediated synergism on the pS2 promoter

Thus, the transcriptional rate of the pS2 promoter is influenced in a variety of ways. The promoter context, i.e an intact AP1 response element adjacent to the ERE in the pS2 promoter, had a significant effect on the potentiation of the pS2 gene expression by the p160 coactivator SRC-1. In addition, the type of p160 coactivator had a clear effect on the transcriptional rate, TIF-2 being a significantly more potent activator of the pS2 gene than SRC-1. Furthermore, TIF-2 was less dependent on the presence of an intact AP1 response element in the pS2 promoter. Finally, also the type of ERE appears

to determine the relative dependence on the promoter context, the consensus EREvit being relatively less dependent on the adjacent AP1 motif as compared to the natural ERE of the pS2 promoter.

Conclusions and future perspectives.

In this thesis evidence is presented showing that SERMs stimulate the SHBG gene expression through a non-ER dependent mechanism. Both tamoxifen and raloxifene stimulated SHBG expression in the absence of ER. In addition, both compounds displayed an additive effect to estrogen-induced ER-dependent SHBG expression. This observation could be of clinical relevance since the elevated SHBG levels observed in pre-menopausal women on adjuvant tamoxifen treatment may be explained by non-ER dependent tamoxifen agonism.

Although tamoxifen stimulation of SHBG transcription clearly is non-ER dependent and appears to involve PKC-signaling the mechanism of action is not yet understood. The SHBG promoter needs to be characterized in more detail. Hence, an attempt to identify the promoter regions through which estrogen and SERMs stimulate SHBG expression should be performed. This may provide further information, e.g. putative response elements, which may contribute to elucidation of the molecular mechanisms that are involved in the stimulation of SHBG gene expression by SERMs in the absence of ER.

Furthermore, it was concluded that a variety of estrogenic compounds display an ERα- or ERβ-selective character in our cell-based transcription assay. Tamoxifen and raloxifene show agonistic activity via ERα but not via ERβ. In addition, raloxifene displays ERα selectivity both with respect to binding affinity and activity in the reporter cells. These studies were performed using the ERβ 485. Presently, however, ERβ 530 has been recognized as the full-length protein. Nevertheless, subsequent studies have revealed that ERβ 530 responds very similarly to the ligands we investigated in our study using ERβ 485.

We also showed that the transcriptional activity of the pS2 gene in response to estrogen is mediated through a crosstalk between an ERE and a nearby AP1 response element in the pS2 promoter. In contrast, the ligand independent stimulation of the pS2

gene by PMA is mainly mediated via the AP1 reponse element. Nevertheless, the presence of ERα, but not of an intact ERE, dramatically potentiated the PMA effect on pS2 gene transcription.

The p160 coactivators, SRC-1 and TIF-2, displayed estrogen-induced synergistic activity that was mediated via the ERE and AP1 response element in the pS2 promoter. Moreover, suppression of pS2 promoter activity using dominant negative fragments of SRC-1 suggested that SRC-1 may function as a bridging factor between the ERE and the AP1 response element. We found that TIF-2 was a more potent activator of the pS2 promoter; however, TIF-2 was less dependent on an intact AP1 response element than SRC-1. In addition, the type of ERE in the pS2 promoter influenced the potentiation by SRC-1, as supported by the less pronounced dependence on the AP1 motif when the natural ERE was substituted for by a consensus ERE. Thus, the estrogen-induced transcriptional response of the pS2 promoter is determined by several parameters which include the p160 coactivator subtype, the promoter context in the vicinity of the ERE as well as the type of ERE present in the promoter.

The fact that both SRC-1 and TIF-2 mediate their synergistic activity via the ERE and the AP1 motif in the pS2 promoter, suggests that they play similar mechanistic roles. However, our studies have revealed differences in their effect on the pS2 promoter. Thus, further studies are required to determine whether also TIF-2 has the capacity to interact with the components of the AP1 complex. If this is the case the interaction surfaces should be mapped.

A variety of questions with regard to pS2 promoter activation could probably be addressed using chromatin immunoprecipitation assays (ChIP). This method provides an outstanding tool for detailed analysis of protein-DNA and protein-protein interactions in the native chromatin context. Furthermore, this technology has been successfully applied to study NR-coactivator interactions (Shang and Brown, 2002; Shang et al., 2000). By the use of ChIP assay it would be possible to elucidate what impact the presence of intact ERE or AP1 response elements in the pS2 promoter has on recruitment of cofactors to the promoter. In addition, putative differences between estrogen and PMA with respect to coactivator recruitment to the pS2 promoter could be investigated.

Finally, it would be of interest to assess the relative roles of the different members of the p160 coactivator family in pS2 promoter activation at endogenous, physiological

coactivator levels. This could be achieved by antisense oligonucleotide technology that reduces the endogenous expression of a specific coactivator.

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