Molecular Basis of Estrogenic Endocrine Disruptor-Estrogen Receptor Interactions: A Comparison Among Species

By

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Abstract

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In recent years, there has been heightened concern that environmental exposure to estrogen mimicking chemicals, known as estrogenic endocrine disruptors (EEDs) may cause adverse health effects in humans and wildlife. Many of the effects of EEDs are mediated through the estrogen receptor (ER). Although the physiological actions of the ER are conserved among species, variation within the amino acid sequence of ligand binding domains suggests that species may exhibit different responses and sensitivities to EEDs.

Species-specific responses to EEDs were first examined in competitive binding assays using glutathione-S-transferase (GST)-ER fusion proteins from several different species. Fusion proteins consisted of the ER D, E, and F domains of human alpha (GST-hERαdef), mouse alpha (GST-mERαdef), chicken (GST-cERdef), green anole (GST-aERdef) and rainbow trout ERs (GST-rtERαdef). Although, the fusion proteins exhibited similar binding preferences for many EEDs, several differences were observed. The GST-rtERαdef, which has the greatest amino acid sequence variability in its ligand

binding domain compared to hERαdef, exhibited the most striking differences compared to the other GST-ERs. The ability of several of these EEDs to induce gene expression mediated by the various ERs was then examined in MCF-7 cells transiently transfected with Gal4-ERdef chimeric receptors. Overall, the data in the gene expression assay correlated with the competitive binding results. However, there were examples where EEDs bound to GST-ERs but were unable to significantly induce ER-mediated gene expression. Intriguingly, the E2-induced response mediated by Gal4-rtERαdef was 2 orders of magnitude lower compared to the other receptors examined. Much of this effect was due to temperature, since when compared to hERα the 280-fold difference at 37°C was reduced to only 9-fold at 20°C. A comparison of their ligand binding pockets identified two conservative amino acid substitutions in rtERα (M317, I496) and hERα (L349, M528). The effect of these substitutions on ligand binding and transactivation was examined by constructing reciprocal mutants. The rtERαdef M317L:I496M mutant exhibited a hERα phenotype with increased E2 binding affinity and transactivation ability at higher temperatures. The hERα L349M:M528I mutant also exhibited a modest trend towards adopting the rtERα phenotype. The lack of a complete exchange of phenotypes indicates that factors outside of the ligand binding pocket are also involved.

Taken together these results demonstrate that ERs from different vertebrate species exhibit different affinities and transactivation responses to EEDs. Since few differences were observed, these data do not preclude the use of a single surrogate ER to examine estrogenic responses for all vertebrate species. The present report also highlights the impact of temperature when comparing functional characteristics of proteins from poikilothermic species, such as rainbow trout, and humans.

Acknowledgements

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LIST OF ABBREVIATIONS

AF-1	Ligand-independent activation function 1
AF-2	Ligand-dependent activation function 2
AR	Androgen receptor
ACTH	Adrenocorticoid hormone
CBP	cAMP responsive element-binding (CREB)-binding protein
COUP-TF	Chicken ovalbumin upstream promoter
DBD	DNA binding domain
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DES	Diethylstibestrol
D-box	Distal-box
DR	Direct repeat
DRIP	VDR interacting protein
EDC	Endocrine disrupting chemical
EDSTAC	Endocrine disruptor screening and testing advisory committee
E2	17β-estradiol
ER	Estrogen receptor
EC_{50}	50% maximal response concentration
ERE	Estrogen response element
EED	Estrogenic endocrine disruptor
E1	Estrone
E3	Estriol
ERR	Estrogen-related receptor
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GST	Glutathione-S-transferase
HAT	Histone acetylase transferase
HDAC	Histone deacetylase
HO-PCB	Hydroxylated polychlorinated biphenyl
IC_{50}	50% inhibitory concentration
LBD	Ligand binding domain
LH	Leutenizing hormone
MAPK	Mitogen-activating protein kinase
NGFI-B	Nerve growth factor-inducible gene B
NCoR	Nuclear receptor corepressor
NR	Nuclear receptor
PBP	PPAR-binding protein
pCAF	p300/CBP associated factor
PCB	Polychlorinated biphenyl
PPAR	Peroxisome proliferator activated receptor
PAS	Per ARNT Sim
P-box	Proximal-box

RXR	Retinoid X receptor
RAR	Retinoic acid receptor
SRC	Steroid receptor coactivator
SERM	Selective estrogen receptor modulator
SMRT	Silencing mediator for retinoid and thyroid-hormone receptor
SHP-1	Small heterodimer partner
TRAP	Thyroid hormone receptor-associated protein
TR	Thyroid receptor
VDR	Vitamin D receptor
WT	Wild-type

CHAPTER 1

Objectives

Rationale

Estrogens regulate several diverse physiological responses including reproductive development, cardiovascular function, and bone metabolism. Estrogens have also been implicated in a number of human disease states, including breast and endometrial cancers (1), cardiovascular disease (2), osteoporosis (3), and Alzheimer's disease (4).

In recent years, it has been suggested that exposure to chemicals in the environment with estrogen-mimicking activities may cause adverse health effects in humans and wildlife (5). These chemicals, known as estrogenic endocrine disruptors (EEDs), encompass a wide range of structurally diverse chemicals including natural products, industrial chemicals, pharmaceuticals, and environmental pollutants. The actions of EEDs are mediated through their binding to the estrogen receptor (ER), though additional mechanisms of action cannot be discounted (6-13). The ER is a member of the nuclear receptor superfamily, a family of ligand- and non-ligand-regulated transcription factors that modulate target gene expression. Even though the physiological actions of the ER are conserved among different species, amino acid sequences of ligand binding domains are variable. A limited number of studies have shown significant differences in ligand preference, binding affinity, and transactivation activity in response to EED exposure among vertebrate species (14-19).

Hypothesis

ERs among different classes of vertebrate species exhibit differences in ligand preference, binding affinities, and transactivation responses to EED exposure due to variation in the amino acid sequence within their ligand binding domains.

Aims

1. Examine and compare differences in ligand preferences and binding affinities of representative ERs from different classes of vertebrate species for EEDs using a competitive binding assay.

2. Investigate the ability of EEDs to induce gene expression mediated by representative ERs from different classes of vertebrate species using transiently transfected MCF-7 human breast cancer cells.

3. Identify amino acid residues putatively involved in ligand binding and perform site-directed mutagenesis to verify the role of these residues in the differential respones observed in Aims 1 and 2.

4. Purification of rainbow trout ERα for further biophysical characterization.

References

1. Bernstein, L., Ross, R. K., and Henderson, B. E. (1992) *J Natl Cancer Inst Monogr* **12,** 137-47

2. Chae, C. U., Ridker, P. M., and Manson, J. E. (1997) *Thromb Haemost* **78**(1), 770-80.

3. Battistini, M. (1997) *Rev Rhum Engl Ed* **64**(6 Suppl), 44S-49S.

4. Birkhauser, M. H., Strnad, J., Kampf, C., and Bahro, M. (2000) *Int J Geriatr Psychiatry* **15**(7), 600-9.

5. Colborn, T., vom Saal, F.S., Soto, A.M. (1993) *Environ. Health Perspect.* **101,** 378-384

6. Adlercreutz, H., Hockerstedt, K., Bannwart, C., Bloigu, S., Hamalainen, E., Fotsis, T., and Ollus, A. (1987) *J Steroid Biochem* **27**(4-6), 1135-44

7. Brouwer, A., and van den Berg, K. J. (1986) *Toxicol Appl Pharmacol* **85**(3), 301-12.

8. Hodgert Jury, H., Zacharewski, T. R., and Hammond, G. L. (2000) *J Steroid Biochem Mol Biol* **75**(2-3), 167-76.

9. Loomis, A. K., and Thomas, P. (2000) *Biol Reprod* **62**(4), 995-1004.

10. Revelli, A., Massobrio, M., and Tesarik, J. (1998) *Endocr Rev* **19**(1), 3-17.

11. Blumberg, B., Sabbagh, W., Jr., Juguilon, H., Bolado, J., Jr., van Meter, C. M., Ong, E. S., and Evans, R. M. (1998) *Genes Dev* **12**(20), 3195-205.

12. Masuyama, H., Hiramatsu, Y., Kunitomi, M., Kudo, T., and MacDonald, P. N. (2000) *Mol Endocrinol* **14**(3), 421-8.

13. Moore, J. T., and Kliewer, S. A. (2000) *Toxicology* **153**(1-3), 1-10

14. Le Drean, Y., Kern, L., Pakdel, F., and Valotaire, Y. (1995) *Mol. Cell. Endocrinol.* **109,** 27-35

15. Connor, K., Ramamoorthy, K., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A., and Balaguer, P. (1997) *Toxicol Appl Pharmacol* **145**(1), 111-23

16. Vonier, P. M., Guillette, J., L.J., McLachlan, J. A., and Arnold, S. F. (1997) *Biochem Biophys Res Comm* **232**(2), 308-312

17. Fitzpatrik, D. W., Picken, C. A., Murphy, L. C., and Buhr, M. M. (1989) *Comp Biochem Physiol* **94C**(2), 691-694

18. Lutz, I., and Kloas, W. (1999) *Sci Total Environ* **225**(1-2), 49-57

19. Petit, F., Valotaire, Y., and Pakdel, F. (1995) *Eur J Biochem* **233**(2), 584-92

CHAPTER 2

Literature Review

Hormones and Cellular Regulation

Hormones are non-nutritive substances that are released into the intercellular environment and act as information carriers or messengers causing changes in cellular stimulation. There are three types of cellular stimulation mechanisms: endocrine, paracrine, and autocrine. Endocrine hormones are released into the bloodstream and carried to distant target sites. In addition to long-range signaling, most cells release chemical signals into the extracellular fluid that influence neighboring cells (paracrine stimulation), though many cells also release chemical signals that modulate their own function, a process known as autocrine stimulation. Frequently, the same hormone can be involved in all three types of cellular stimulation paradigms.

The initial step in which hormones exert a response involves their interaction with specific receptors. Hormones like FSH (follicle-stimulating hormone), LH (luteinizing hormone), insulin, somatotrophin and others bind to transmembrane receptors on the cell surface that initiate a signal transduction cascade resulting in changes a number of intracellular events, including the release of second messengers as well as the activation of kinases (1). This leads to a very rapid response that is quickly attenuated once the cellular equilibrium is re-established. Steroid hormones such as estrogens, androgens, and glucocorticoids and the thyroid hormone diffuse into the cell and bind with high affinity to their cognate receptors. Liganded receptors either translocate to the nucleus or are already present in the nucleus where they bind DNA, causing dose-dependent changes in transcription. Since changes at the transcriptional level are relatively slow, these compounds produce more long-term cellular effects compared to peptide hormones that mediate their effects through a signal transduction pathway. There is increasing

evidence that in addition to leading to changes in gene expression, estrogens are also able to bind to membrane receptors leading to induction of a signal transduction cascade (2).

The actions of estrogens are regulated through negative and positive feedback mechanisms on the hypothalamic-pituitary-gonadal axis (Figure 1). The synthesis and release of estrogen from the ovaries is synchronized by the pituitary gonadotropins FSH and LH, which are regulated by the release of hypothalamic gonadotropin-releasing hormone (GnRH). GnRH concentrations are influenced through negative and positive feedback by steroid hormones. Adrenocorticoid hormone (ACTH) released from the pituitary acts on the adrenal glands to cause the synthesis and release of androgens, which become converted to estrogen by the enzyme aromatase. Estrogens as well as progesterone can also act directly on the pituitary to decrease FSH and LH. Moreover, the ovarian protein, inhibin, also down regulates FSH synthesis (1).

Estrogen Receptor Agonist and Antagonist Ligands

Estrogens play a critical role in growth, development, and maintenance of a number of cellular functions in all vertebrate species (3). The three natural estrogens are 17β-estradiol (E2), estrone (E1), and estriol (E3), though there are a number of metabolic derivatives (Figure 2). E2 is the most potent of the three and the primary estrogen in mammals and non-mammalian vertebrates (3). In addition to the role estrogens play in reproductive physiology, estrogens can also induce the growth and proliferation of cancer cells. As a result considerable effort has been put forth in developing antiestrogen drugs for the treatment and prevention of breast cancer (4). Ligands such as E2 and the Figure 1

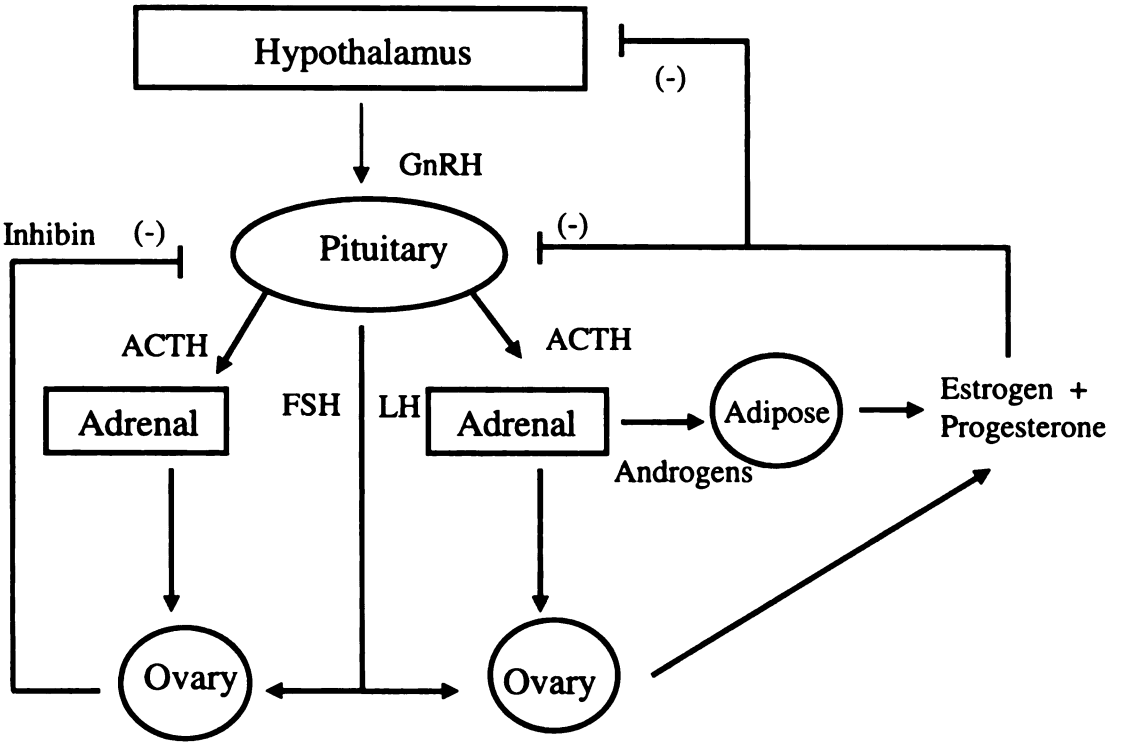


Figure 1. Negative and positive feedback regulation of estrogens and progesterone on the hypothalamic-pituitary-ovarian axis. Arrowheads denote positive regulation. GnRH, gonadotropin-releasing hormone, ACTH, adrenocorticotropic hormone, LH, luteinizing hormone, FSH, follicle stimulating hormone.

synthetic nonsteroidal estrogen diethylstilbestrol (DES) function as pure agonists, whereas others such as ICI 182,780 function as pure antagonists (Figure 2), though it has been reported to function as a partial agonist in the sheep uterus (5). The synthetic drugs, raloxifene and tamoxifen (6), are routinely used in the treatment of breast cancer, and belong to a class of compounds known as selective estrogen receptor modulators (SERMs) (Figure 2). The antagonistic and agonistic activities of these compounds are tissue- and promoter-specific (7,8).

Environmental Endocrine Disruptors

In recent years, there has been heightened concern that exposure to hormonally active chemicals, known as endocrine disrupting chemicals (EDCs) may cause adverse health effects in humans and wildlife (9). EDCs have been defined as any exogenous substances that can cause adverse health effects in an intact organism or its progeny, secondary to changes in endocrine function (10). Suspected EDCs encompass a wide range of compounds including natural products (e.g. phytoestrogens, mycotoxins), environmental pollutants (e.g. polychlorinated biphenyls, dioxin), pharmaceuticals, pesticides and industrial chemicals.

Some effects attributed to EDC exposure include: increased susceptibility to hormone-dependent cancers (11,12), reproductive tract abnormalities (13), compromised reproductive fitness (14), and neurological (15) and developmental abnormalities (16,17). In response, new legislation was implemented in the form of the Food Quality Protection Act (1996 - Bill number P.L. 104-170) as well as amendements to the Safe Drinking Water Act (05 1996 - Bill number S.1316) that require the United States Environmental

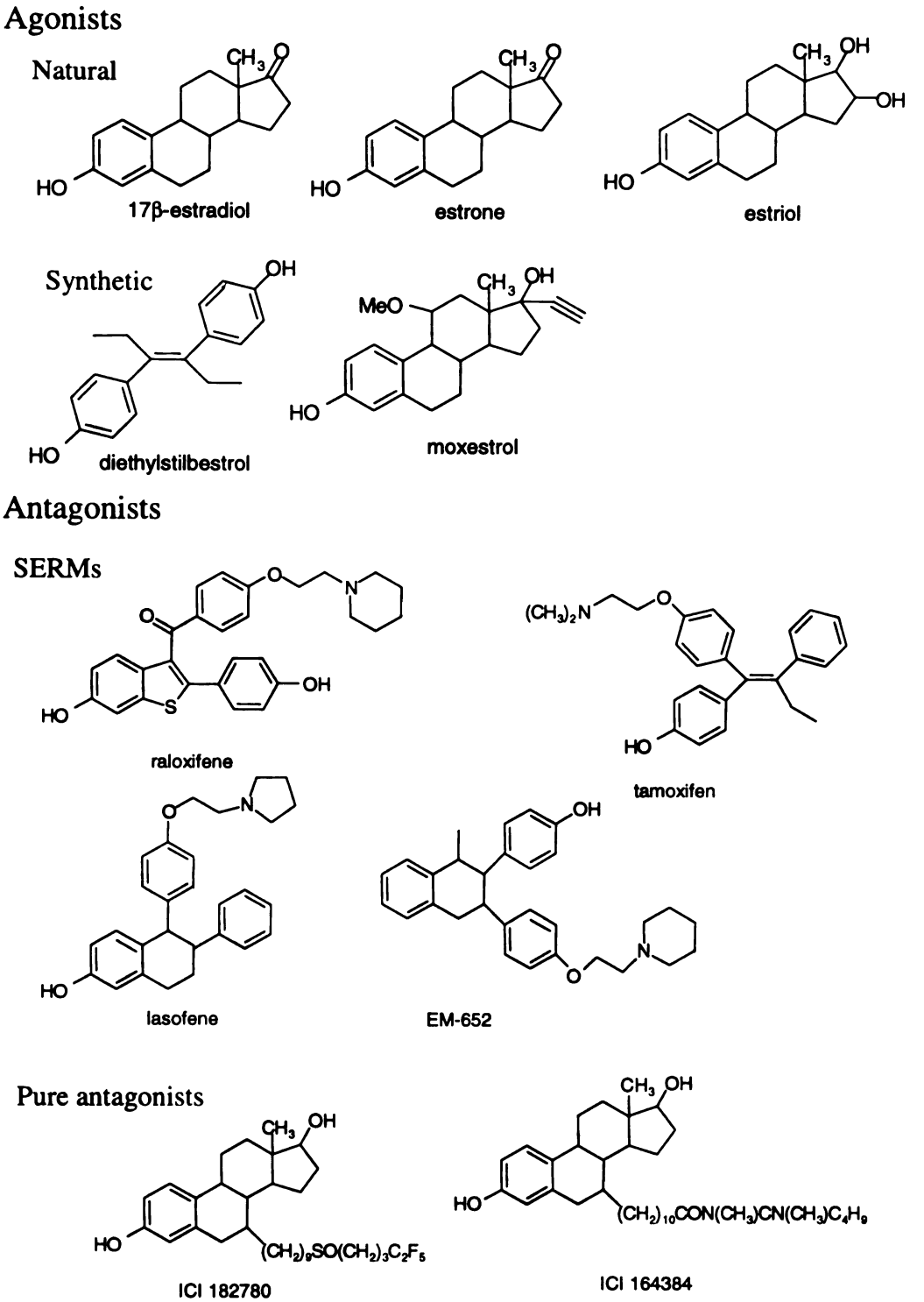


Figure 2. Estrogenic (agonist) and antiestrogenic (antagonist) ligands of the ER. SERMs, selective estrogen receptor modulators.

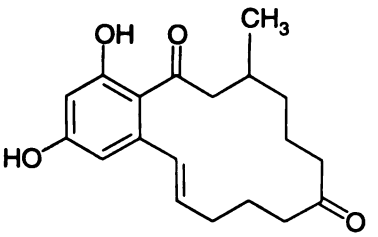
Protection Agency (US EPA) to screen and test chemicals for estrogenic, androgenic and thyroid-like activities. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was established and charged with the development and implementation of a screening and testing strategy (18). EDSTAC deliberations resulted in recommendations for tiered strategy consisting of prioritization followed by screening and testing approaches involving a combination of in vitro and in vivo assays. It has been estimated that more than 30,000 chemicals require testing.

Much attention has focused on estrogenic endocrine disruptors (EEDs) (16,19-21). EEDs encompass a wide range of compounds including natural products, environmental pollutants, pharmaceuticals and industrial chemicals (Figure 3). Many of these agents do not share any obvious structural similarity to the prototypical estrogen, E2, which makes identification based solely on molecular structure difficult (22).

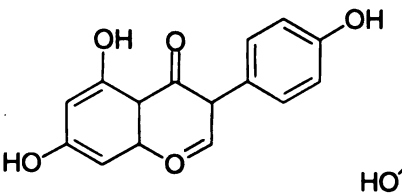
Although controversial, there are reports of a decrease in human sperm production and seminal volume during the past half-century (23-25). However, reproductive abnormalities have been observed in mammals (26), reptiles (17,27), birds (28,29) and several fish species (30) following exposure to environmental contaminants. The feminization of gull embryos (31) and gonadal sex reversal of turtles (32,33) have also been reported following exposure to 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) and polychlorinated biphenyls (PCBs), respectively. Induction of vitellogenin, a gene normally induced in egg laying females, has been detected in male fish located downstream of sewage treatment effluent (34).

It has been proposed that the activity of estrogenic, as well as androgenic and thyroid-like chemicals is mediated by their respective nuclear receptors (NRs) (35),

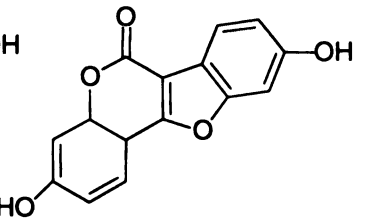
Natural



zearalenone

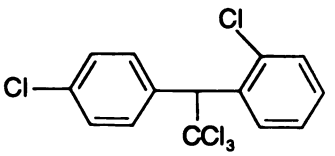


genistein

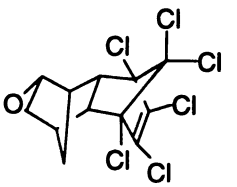


Coumestrol

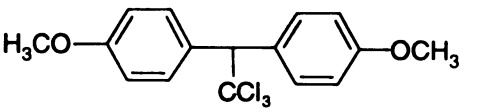
Pesticides



o,p`-DDT

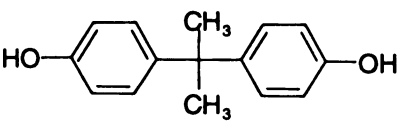


Dieldrin

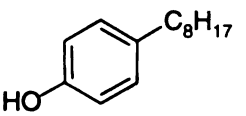


Methoxychlor

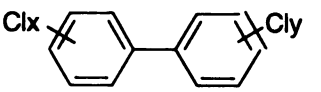
Industrial Chemicals/Contaminants



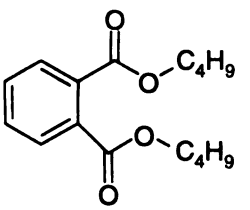
Bisphenol A



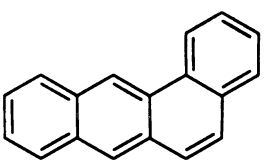
octylphenol



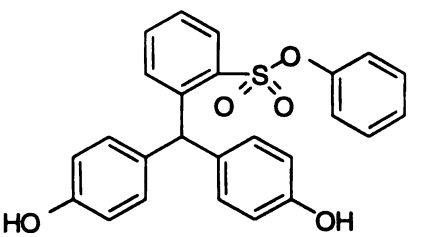
Polychlorintated biphenyl



Dibutyl phthalate



benz[a]anthrecene



Phenol Red impurity

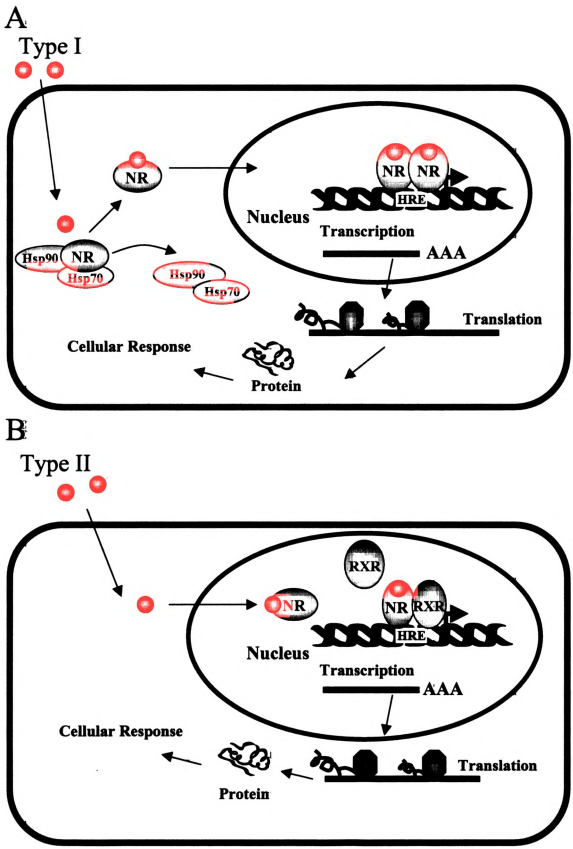
Figure 3. Suspected estrogenic endocrine disruptors (EEDs) and their classifications.

though other mechanisms such as interactions with binding globulins (36,37), inhibition of aromatase, the enzyme that converts testosterone to estrogen (38,39), and binding to membrane receptors (40,41) or other NRs (42,43) cannot be excluded.

Nuclear Receptors

NRs are a superfamily of ligand- and non-ligand-regulated transcription factors that play critical roles in development, differentiation, and homeostasis. They regulate gene expression through binding small hydrophobic ligands, such as steroid and thyroid hormones, vitamin D, and retinoids. These proteins represent the largest family of transcription factors known; yet relatively low amounts of these receptors (10^3-10^4 copies/cell) are present in a cell (35). This superfamily can be divided into four broad categories based on their ligand binding, dimerization abilities, cellular distribution, and DNA binding properties. The type I NRs include receptors for steroids such as estrogens, androgens, progestins, glucocorticoids, and mineralcorticoids. These receptors are bound to heat shock proteins and sequestered in the cytoplasm in the absence of ligand. Ligand binding causes the dissociation of heat shock proteins and translocation to the nucleus where they bind their cognate palindromic response elements as homodimers (Figure 4A). The ER, unlike other type I NRs, is found exclusively in the nucleus, a behavior that has been confirmed in the presence and absence of ligand (44,45). The type II NRs include among others, receptors for thyroid (TR), vitamin D (VDR), and all-trans retinoic acid (RAR). These receptors are found solely in the nucleus and bind to direct repeat response elements as heterodimers with 9-cis retinoid X receptor (RXR) (45) (Figure 4B). The type III and type IV NRs contain a large number of putative receptors for

Figure 4. Proposed mechanism of action of (A) class I and (B) class II nuclear receptors. See text for details. NR, nuclear receptor; hsp90, heat shock protein 90; hsp70, heat shock protein 70; HRE, hormone response element; RXR, retinoid X receptor. This figure is presented in color.


A
Type I
NR
NR
NR
HRE
Nucleus
Transcription
AAA
Hsp90
NR
Hsp70
Hsp90
Hsp70
Translation
Cellular Response
Protein
B
Type II
RXR
NR
NR
RXR
HRE
Nucleus
Transcription
AAA
Cellular Response
Translation
Protein

which ligands have not been identified or may not exist, and are referred to as orphan receptors (46,47). These putative receptors have been identified based on their sequence homology to type I and type II NRs. Type III members include: peroxisome proliferator activated receptor (PPAR) (48), chicken ovalbumin upstream promoter transcription factor (COUP-TF) (49), hepatic nuclear factor-4 (50), and several others (47,51). These putative receptors bind to DNA as homodimers, and/or heterodimers with RXR. Type IV NRs, which are also classified as orphan receptors include: estrogen-related receptor (ERR) (52), nerve growth factor-inducible gene B (NGFI-B) (53), SF-1 (54), and many others (47,51). Type IV members bind DNA exclusively as monomers, a feature that distinguishes them from other NRs. Moreover, the orphan receptor DAX-1 contains a unique three repeat domain in the N-terminus, which represents a novel type of single stranded DNA/RNA-binding domain (55). Short heterodimerization partner (SHP) is an unusual orphan receptor that lacks a conventional DNA binding domain (DBD) but contains a putative central ligand binding domain (56). SHP interacts with a variety of NRs including RXR and several of its heterodimerization partners and acts as a negative regulatory of ER function (57). Orphan receptors have attracted considerable interest since they could help uncover novel endocrine regulatory systems.

Most NRs display a modular structure with five to six distinct regions, termed A-F domains and contain two separate activation functions (Figure 5). The N-terminal A/B regions contain a ligand-independent activation function (AF-1), which can constitutively activate transcription. The C region contains the DBD, which is characterized by two

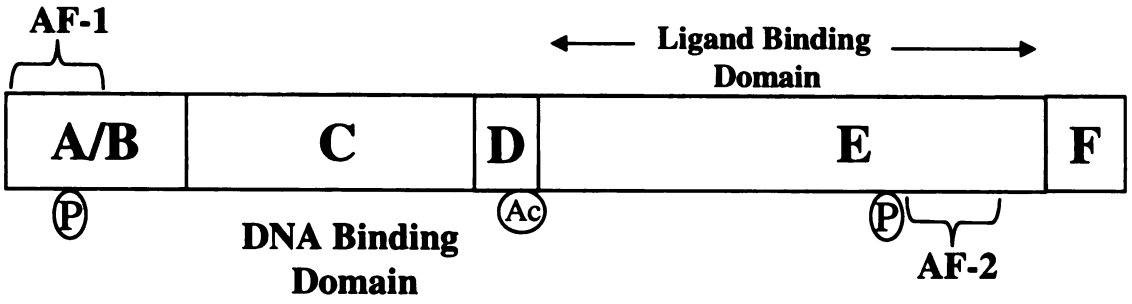


Figure 5. Conserved domain structure of nuclear receptors. A schematic representation of the human estrogen receptor α is shown. AF-1 and AF-2 refer to the ligand independent and ligand dependent transactivation domains, respectively. The encircled P refers to known sites of phosphorylation, while the encircled Ac identifies sites of acetylation.

zinc fingers and recognizes cognate cis elements in the upstream regulatory region of target genes, referred to as response elements (Figure 6A). The proximal-box (P-box) located in the first zinc finger interacts with DNA and is involved in binding site discrimination (58,59), whereas the distal-box (D-box) located in the second zinc finger supports receptor dimerization (59-61).

The nature of the response element varies among the different types of NRs. NR response elements for type I receptors consist of inverted palindromic sequences, which are separated by a three nucleotide spacer (Figure 6B). Type II NR response elements are generally direct repeats (DR) composed of a minimal hexanucleotide core spaced by 1 to 5 nucleotides (Figure 6B). A flexible hinge or D region separates the DBD from the E region, which contains the ligand binding domain (LBD), dimerization interface, and the ligand-dependent activation function, AF-2. Recent studies have demonstrated that the hinge region may serve as a docking site for corepressor binding (62,63). Acetylation of lysine residues within the hinge region of the ER has recently been shown to influence ligand sensitivity as well as recruitment of corepressor complexes (64). The activities of AF-1 and AF-2 vary depending on the promoter (65) and cell type, and in some cases both are required for full transcriptional activation (66). Several receptors also contain a variable C-terminal F domain, which has been shown to influence interactions with coactivators (67,68).

X-ray structures of the LBD reveal that NR LBDs exhibit a similar overall architecture with the LBD folded into a three-layered antiparallel α-helical sandwich comprising a central core layer of helices (H5, H6, H9, and H10) found between two additional layers of helices (H1-4 and H7, H8, and H11) (69-76) (Figure 7). Biochemical

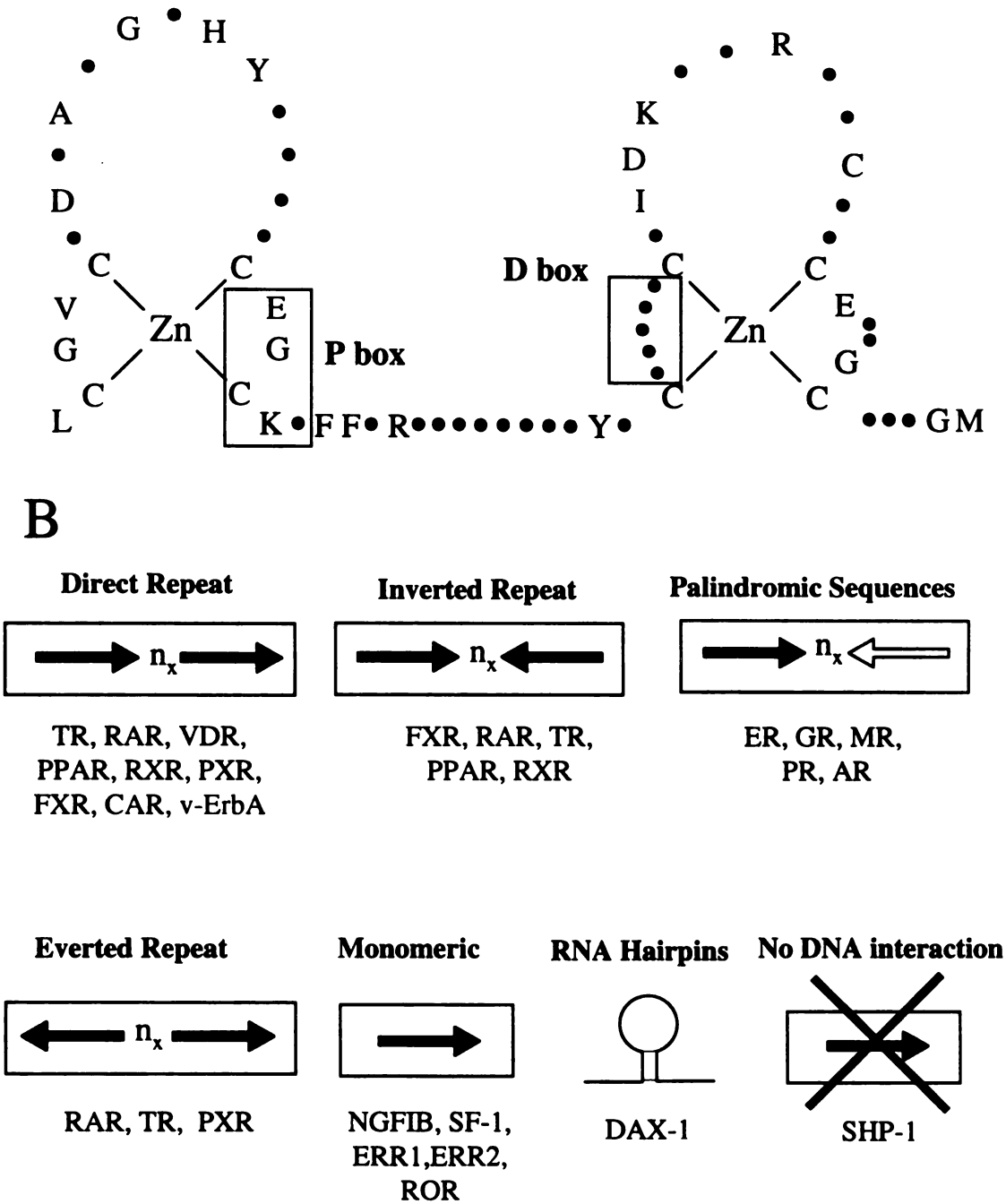


Figure 6. (A) Hypothetical "zinc fingers" in the DNA binding domain of nuclear receptors. Conserved amino acid residues are shown, whereas amino acid residues for which no consensus exists, are represented by a closed circle. Residues which make up the P (proximal) box and D (distal) box are boxed. (B) Different types of hormone response elements and DNA interactions among nuclear receptors.

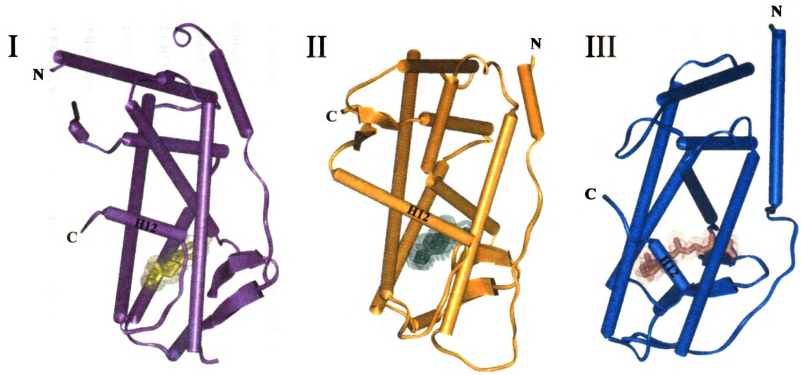


Figure 7. Comparison of the conserved conical fold of nuclear receptor ligand binding domains. (I) Estrogen receptor α complexed with 17β-estradiol (73), (II) androgen receptor complexed with dihydrotestosterone (76), and (III) retinoic acid receptor complexed with all-trans retinoic acid (70). Nuclear receptor ligand binding domains are folded into a three-layered antiparallel α-helical sandwich. This fold creates a sizeable binding cavity of which only 59% to 67% is occupied by ligand. Helix 12 (H12), which corresponds to the core of the AF-2 activation domain is positioned over the ligand binding cavity. This precise positioning of H12 has been proposed as a prerequisite for transcriptional activation and helps form the docking site for LxxLL containing coregulator proteins. This figure is presented in color.

and structural studies have demonstrated that a substantial conformational change occurs after ligand binding (77,78). Comparison of the apo-RXR and 9-cis retinoic acid bound RXR crystal structures confirms the existence of a ligand-induced conformational change, which triggers the receptor to form an activated complex and expose amino acid residues within AF-2 (helix 12), which are necessary for interaction with coactivators (78). The main conformational change occurs in the N-terminal regions of helices 3, 11, and 12 (Figure 8). This movement causes hydrophobic residues previously exposed to solvent to be directed towards the ligand binding cavity, while at the same time allows other hydrophobic residues to form a protein surface necessary for coregulator interaction (78).

Estrogen Receptor α, β, and γ

Many of the effects of estrogen are mediated through its binding to the ER and subsequent alterations in gene expression, though non-genomic effects have also been reported (2). Two ER isoforms exist, ERα and ERβ, each one is encoded from a distinct gene and displays tissue-specific distribution and differential ligand preference (79,80). A comparison of the amino acid sequence identity between the two isoforms reveals that the most conserved region is the DBD (97%), whereas the NH_2-terminal A/B domains and LBD are only 20% and 60% identical in amino acid sequence (81). Although both isoforms exhibit similar affinity for E2, a comparison of their tissue distributions suggests that they may have distinct biological roles (81). Several groups have shown that ERα and ERβ form functional heterodimers in vitro and in vivo (82-84). Interestingly the isoforms, the heterodimers would predominate (81,83). The physiological role of each

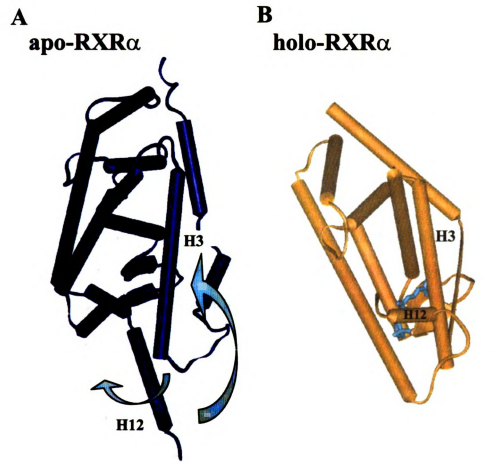


Figure 8. The ligand induced conformational change of human retinoid X receptor (RXR). Comparison of the (A) unliganded (apo-RXRα, shown in blue) and liganded (holo-RXRα shown in orange). The main conformational differences were observed in helices 3 (H3) and H12 following ligand binding. The arrows emphasize the ligand-induced conformational change. The ligand, 9-cis retinoic acid, is shown in light blue. This figure is presented in color

isoform is being intensely studied through the generation of isoform-specific knockout mice (85-87).

The DBDs of ERα and ERβ, like other NRs, allow the receptors to bind to their cognate DNA target sequence, referred to as an estrogen responsive element (ERE). It has been demonstrated that both ER isoforms do not signal only through binding EREs but can also signal through AP-1 and Sp-1 elements (88-90). Intriguingly, ERβ exhibited distinct properties from ERα on both AP-1 and Sp-1 elements. Antiestrogen binding to ERβ caused an increase in reporter gene expression, whereas binding to ERα resulted in an opposite effect examined under the same conditions (88,90)

The recent discovery of a third unique type of ER, ERγ, isolated from Atlantic croaker and zebra fish, has amplified the complexity of estrogen signaling (91). Phylogenetic analysis revealed that ERγ arose from a relatively recent gene duplication of ERβ (91). Teleost (bony fish) genomes have been shown to support significantly more gene duplications than their mammalian counterparts (92). Therefore, ERγ may not represent a third unique ER isoform and may simply represent a genetic duplication of ERβ. In addition, ERα and ERβ have been described in several vertebrate species, ERγ has thus far only been reported in teleosts (91).

X-ray structures of several NR LBD complexes with various ligands have provided insight into the molecular basis of agonism and antagonism. Ligand binding is achieved by a combination of specific hydrogen bonding interactions and the hydrophobic nature of the binding pocket. In the ERα-E2 complex, E2 makes direct

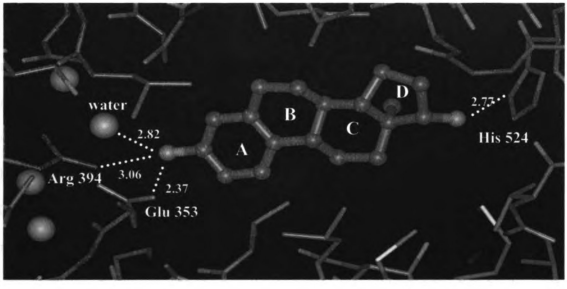


Figure 9. Interactions made by 17β-estradiol in the human estrogen receptor α ligand-binding cavity. This figure was generated in InsightII using the Brookhaven Protein Data Bank entry 1ERE (73). The three amino acids residues (Glu353, Arg394, and His524) that participate in direct hydrogen bonds with the ligand and the distances of the interactions are shown. This figure is presented in color.

hydrogen bonds with the side chains of glutamate 353, arginine 394 and histidine 524. Hydrophobic interactions further stabilize the ligand in the binding cavity (Figure 9). Interestingly, ligands with agonist and antagonist activity stabilize the LBD in distinct conformations, most notably in the orientation of helix 12. In the E2-liganded complexes, helix 12 is positioned over the ligand binding cavity and forms a hydrophobic groove with helices 3, 4, and 5, as well as a turn between helices 3 and 4 (Figure 10). This hydrophobic groove has been proposed as the AF-2 surface, which is capable of interacting with coactivators (73,93). In the raloxifene-liganded structure, helix 12 is prevented from forming part of the AF-2 interaction surface and instead lies in a groove between helices 3 and 5, in effect blocking the coactivator recognition site and mimicking the interactions formed between the NR box motif and the LBD (73,74,93) (Figure 10). The position adopted by helix 12 in the partial agonist genistein-bound structure only partially mimics the NR-box binding mode (74) (Figure 10). In the pure antagonist bound structure the bulky side chain of ICI 164,384 binds along the NR-box binding site and physically prevents helix 12 from adopting either agonist or antagonist orientation (94), further illustrating the importance of the orientation of helix 12 in ER activity.

In addition to the ligand-induced activation of the ER, phosphorylation has been shown to play a key role in modulating ER activation (95-97). Treatment of cells with growth factors was shown to increase the activity of the ER as well as its phosphorylation state. Subsequent studies demonstrated the serine 118 was phosphorylated and required for full ligand-independent receptor activation (95). Serine 118 was later shown to be a substrate for mitogen-activating protein kinase (MAPK), linking ER activity to the RAS-MAPK pathway (96). ERα is also phosphorylated at tyrosine 537, which when mutated

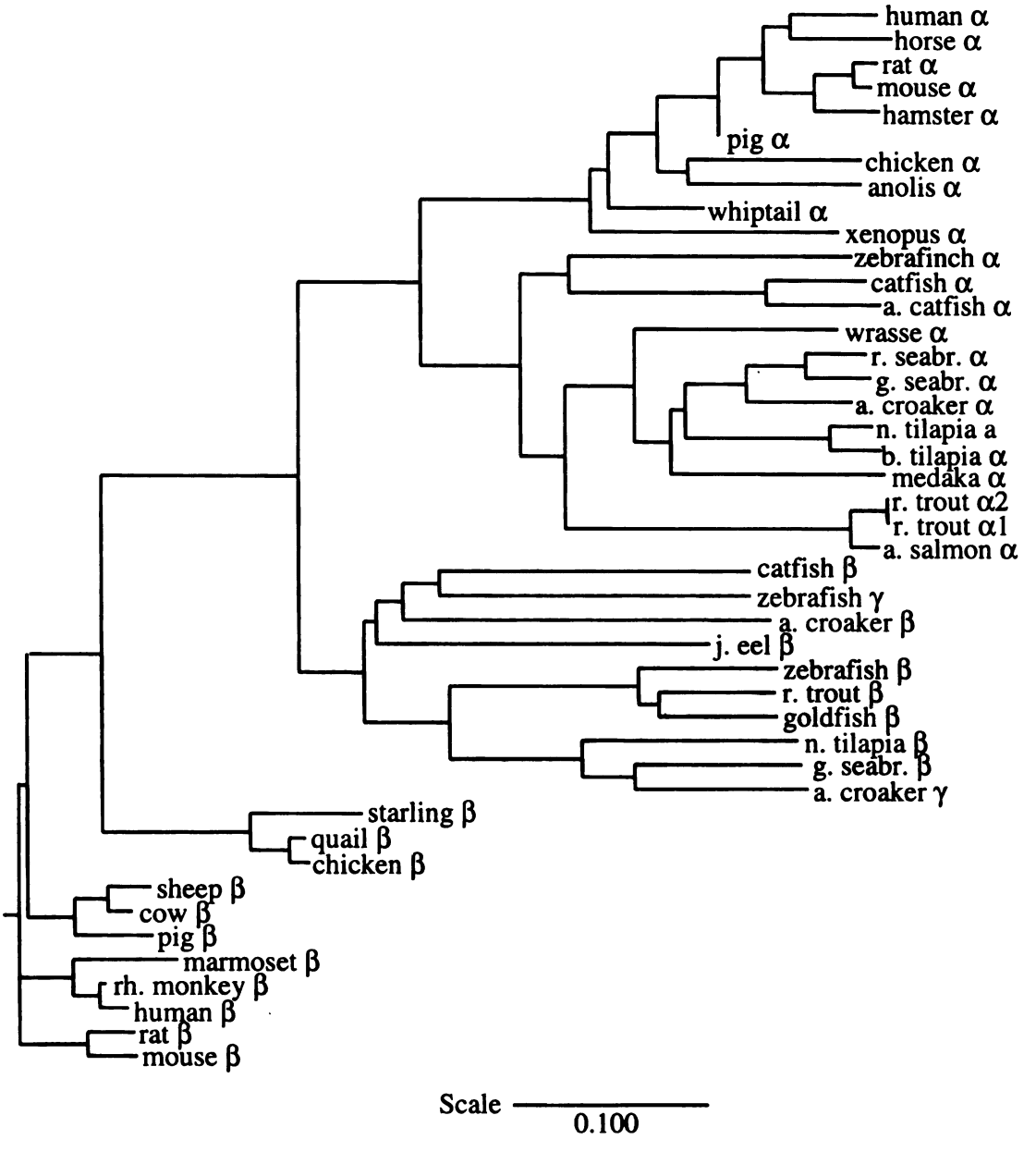
Figure 10. Comparison of the ligand induced orientation of helix 12. Position of helix 12 in the (A) human estrogen receptor α-17β-estradiol bound complex (73), in (B) human estrogen receptor β–genistein bound complex, and (C) rat estrogen receptor-β raloxifene bound complex. This figure was taken from (74) and is presented in color.

causes a ligand-independent increase ER activity (97).

Phylogenetic Comparison of ERs

Phylogenetic analysis reveals that there are distinct ER types. The teleost ERαs and ERβs form distinct groups compared to all other vertebrate ERs (Figure 11). The interspecies homology is higher than the similarity between the α and β isoforms. Interestingly, the two ERγs in the database, cluster with the other teleost ERβs and do not form a separate and distinct branch. A more detailed analysis of the sequence differences among ER D, E, and F domains from several different species, including representative species from 5 different vertebrate classes; mammalian, avian, amphibian, reptilian, and actinopterygii (ray-finned fish) is shown in figure 12. These results demonstrate that significant variability exists within the ligand-binding portion of the different receptors and between α and β isoforms. Despite the differences in degree of sequence identity among species, ERs from all species harbor the same three equivalent amino acids to hERα (glutamate 353, arginine394 and histidine524) that participate in direct hydrogen bonds and stabilize E2 in the binding pocket. (73,74,98). However, differential binding of several natural and synthetic chemicals to hERα and hERβ, as well as to ERs from different species have been reported (see below), suggesting that additional amino acid residues may also play a role in determining ligand preference and relative binding affinity. Despite significant differences in amino acid sequence within ER LBDs a comparison of the amino acid sequences that line the ligand-binding cavity and interact with E2 reveals that few amino acid differences exist among different species compared to hERα. For the aER and xER only single amino acid substitution exist, phenylalanine

Figure 11. Phylogenetic analysis of estrogen receptor (ER) sequences. The distance between nodes reflects the degree of sequence identity when doing pair wise alignments. The value of 0.1 corresponds to a difference of 10% among sequences. This figure was generated using the ClustalW alignment function in MacVector 6.5 (Oxford Molecular Ltd.). GenBank accession numbers: anolis ERα AF095911, African catfish α (a. catfish α) X84743, Atlantic croaker ERα (a. croaker α) AF298183, Atlantic croaker β (a. croaker β) AF298181, Atlantic croaker ER γ (a. croaker γ), Atlantic salmon α (a. salmon α) X89959, blue tilapia α (b. tilapia α) P50240, catfish α AF061275, catfish β AF185568, chicken α X03805, chicken β AB036415, cow β Y18017, gilthead seabream α (g. seabr. α) AJ006039, gilthead seabream (g. seabr. β) AF136980), goldfish β AF061269, hamster α AF181077, horse α AF124093, human α X03635, human β AB006590, Japanese eel β (j. eel β) AB003356, marmoset β Y09372, medaka β AB033491, mouse α M38651, mouse β U81451, Nile tilapia α (n. tilapia α) U75604, Nile tilapia β (n. tilapia β) U75605, pig α AF035775, pig β AF267736, quail β AF045149, rainbow trout α1 (r. trout α1) AJ242740, rainbow trout α2 (r. trout α2) AJ242741, rainbow trout β (r. trout β) AJ289883, rat α Y00102, rat β AJ002602, red seabream α (r. seabr. α) AB007453), rhesus monkey β (rh monkey β) AF119229, sheep β AF177936, starling β AF113513, whiptail α S79923, wrasse α AF326201, xenopus α L20738, zebrafinch α L79911, zebrafish α AF349412, zebrafish β AF349414, zebrafish γ AF349413.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wrasse α
r. seabr. α
g. seabr. α
a. croaker α
n. tilapia a
b. tilapia α
medaka α
r. trout α2
r. trout α1
a. salmon α
catfish β
zebrafish γ
a. croaker β
j. eel β
zebrafish β
r. trout β
goldfish β
n. tilapia β
g. seabr. β
a. croaker γ
starling β
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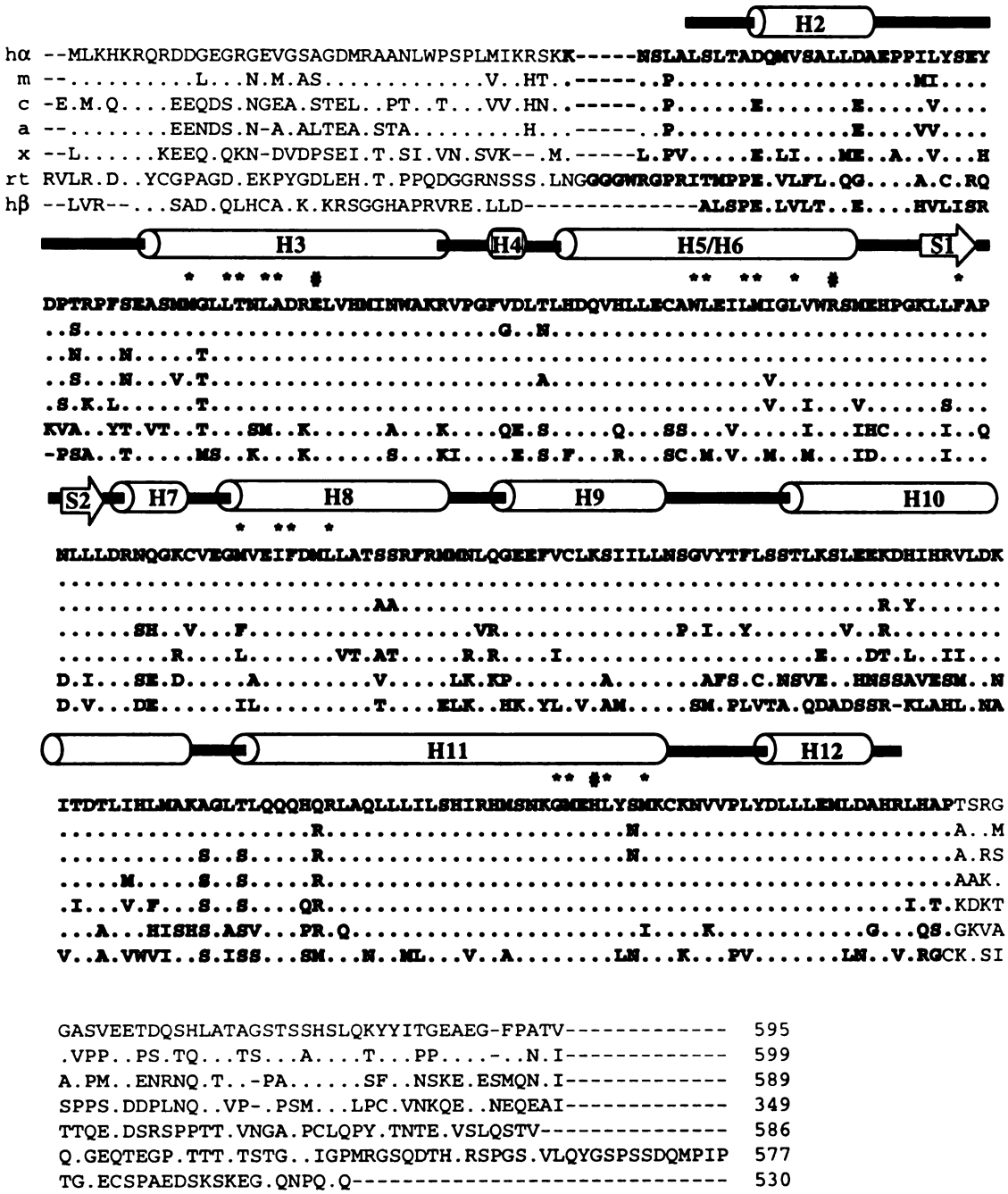

Figure 12. Structural based alignment of the estrogen receptor D, E and F domains of human α (hα), human β (hβ), mouse (m), chicken (c), anolis (a), xenopus (x), and rainbow trout (rtα). Identical amino acid residues are represented as dots while missing residues are shown as dashes. The ligand binding domains (E domains) are shown in bold. Residues that line the hormone binding pocket and that interact with 17β-estradiol are marked with a * and #, respectively. Boundaries of the helices and beta sheets were taken from PDB 1ERE (73).

175 in the aER and leucine 413 are both substituted to methionine 421 in hERα (Figure 12). Two amino acid substitutions exist between hERα and both hERβ and rtERα. In hERβ, methionine 336 and isoleucine 373 are substituted for leucine 384 and methionine 421, respectively, whereas in rtERα methionine 317 and isoleucine 496 are substituted for leucine 349 and methionine 528, respectively (Figure 12). This suggests that these residues may contribute to the observed differences in ligand preferences, binding affinities, and transactivation responses to EEDs among different ERs (see below).

Coregulators

Biochemical and expression cloning studies have identified a number of proteins that interact with NRs in a ligand-dependent and/or ligand-independent manner (99). Many of these proteins have been shown to potentiate NR activity in cell based cotransfection assays, suggesting that they act as coregulators of NR function (99). Coregulators can be classified as coactivators and corepressors. Coactivators lead to an increase in NR activity, whereas corepressor attenuate or inhibit NR activity. Several structurally distinct classes of nuclear receptor coregulators have been identified, some of which are subunits of large multiprotein complexes. For example, the steroid receptor coactivator (SRC) family of coactivators (100) is a family of 160 kDa molecular weight proteins that were the first coregulators shown to interact with NRs. Members of this family include, SRC-1 (also known as p160/NCoA-1/ERAP-160) (100,101), SRC-2 (also known as TIF2/GRIP-1/NCoA-2) (102,103), and SRC-3 (also known as AIB1/ACTR/RAC-3/TRAM-1/pCIP/NCoA-3) (104-108). Once recruited by NRs, these coactivators form a mutiprotein complex with CBP/p300 (105,107) leading to increases

in transcription. SRCs are members of the PAS domain family, which contain a N-terminal basic helix-loop-helix region, followed by PAS A and PAS B domains, and a C-terminal glutamine rich region (109,110). Functional and structural studies have shown that coactivators interact with the AF-2 region via short NR-boxes or LxxLL motifs, where L refers to leucine and x refers to any other amino acid residues, to transduce the ligand signal to the transcriptional machinery (93,111,112). Recent studies have shown that nuclear receptors have distinct affinities for coactivators; for example PPARγ has a greater affinity for CBP than for SRC-1, whereas the ER preferentially binds SRC-1 but exhibits very weak affinity for CBP (113). In addition, the NR affinities of different coactivators and various NR boxes present within coactivators have been demonstrated to exhibit ligand dependent variability (114,115).

Several lines of evidence suggest that the SRC family of coactivators are important in NR function and disease (104,116,117). However, studies reveal potential redundancy in NR coactivator function since only subtle effects in SRC-1 knockout mice were observed, which may have been the result of a compensatory increase in SRC-2 levels (118). In contrast, SRC-3 is required for normal development and female reproductive function, suggesting that it plays a different physiological role than SRC-1 and provides evidence of diversity within the SRC family (119).

Coregulators and NRs have been shown to interact with the general transcription factors CBP/p300 and pCAF. SRC-1, SRC-3, CBP/p300 and pCAF possess histone acetyltransferase (HAT) activity, which disrupts nucleosomes and leads to transcriptional activation (105,120). In the absence of ligand some NRs associate with nuclear receptor

corepressors SMRT (63) and NCoR (62). The association of NCoR with TR is crucial for the ligand independent nuclear retention of TR (121). Both SMRT and NCoR recruit SIN3 and HDACs to form a large corepressor complex that contains histone deacetylase activity (122,123), implicating histone deacetylation in transcriptional repression.

Three models, sequential, combinatorial, and parallel, have been proposed to explain how the extraordinary numbers of coregulators modulate NR transcriptional activation (99). In the sequential model, one of the complexes is recruited to the promoter, which leads to the recruitment of additional proteins. Alternatively, a combinatorial model has been put forth where multiple complexes are present and their combined activity is required for transcriptional activation in vivo. Finally, transcriptional activation may occur in a parallel fashion, with different complexes forming on the same promoter, allowing for tissue or cell specific responses. Recent reports favor the sequential model where different cofactors cycle during the onset and course of transcription (Figure 13) (124). The binding of E2 to the ER causes the exchange of the corepressor complex with a coactivator complex. This is followed by the recruitment of coactivators with HAT activity such as p300/SRC complex and PBP, the protein that anchors the DRIP/TRAP complex to NRs (125). The p300 complex modifies chromatin, but is present only during the initial cycle of transcription. Once the RNA polymerase II C-terminal tail is phosphorylated, CBP replaces the p300 complex causing the recruitment of pCAF. This leads to the concomitant release of SRC and the ER. CBP and pCAF disassemble and the cycle is set to repeat (124).

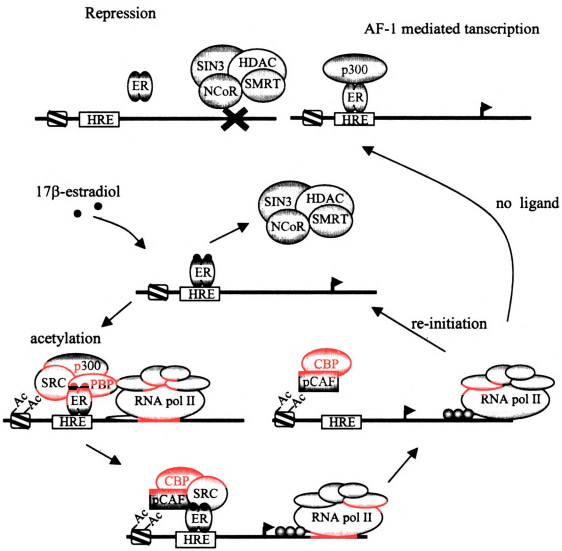

Figure 13. Proposed model for transactivation by ER including interactions with coactivators and corepressors. This model was modified from Shang et al. (124), which demonstrated the sequential and cyclical formation of complexes leading to transcriptional activation in the presence of an agonist such as 17β-estradiol. This figure is presented in color.

Estrogen-Responsive Genes

Several estrogen responsive genes have been characterized in a variety of species. Analysis of the upstream regulatory regions of many estrogen-responsive genes revealed a common sequence motif referred to as an estrogen responsive element (ERE), which was determined to have the following consensus sequence 5'-GGTCAnnnTGACC-3' (126,127). Electromobility gel electrophoresis assays demonstrated that the ER binds with high affinity to consensus EREs as a homodimer (56). The sequences flanking the ERE have also been shown to influence stability of the ER-ERE interaction (128,129). Genes such as Xenopus vitellogenin A2 (126) and human oxytocin (130) contain a single consensus ERE, whereas others like Xenopus vitellogenin B1 (126,131), the human PR (132), human c-fos (133) and human pS2 (134) contain single or multiple copies of EREs, which contain one or more nucleotide differences from the consensus ERE. Some estrogen-responsive genes such as ovalbumin (135) contain multiple copies of ERE-half sites, but not the palindromic sequence. These genes also contain response elements for RXR, RAR, and TR (136,137), suggesting that the cellular concentrations of NRs influence the regulation of target genes. In addition, the identification and study of estrogen-responsive genes is further complicated by the observation that the ER can regulate gene expression through interaction with other transcription factors (88).

Species-Specific Binding Preferences and Transactivation Responses to EEDs

Although the physiological actions of the ER are conserved among different species, the amino acid sequences of the regions involved in ligand binding are quite

variable. This suggests that species may exhibit different responses and sensitivities to EEDs, and that one species may not be an appropriate surrogate for use in identifying and predicting responses in other species. Significant variability exists in the reported binding affinity for E2 among vertebrate ERs (Table 1). Receptor binding studies have shown that E2 exhibits a 5-fold lower binding affinity for the rainbow trout ERα (rtERα) than for the hERα (138,139). When expressed in yeast the rtERα exhibits a 10-fold lower responsiveness to E2 and a significantly reduced affinity for E2 at elevated temperature when compared to hERα (140). Weaker E2 responsiveness of rtERα has also been observed in embryonic salmonid cells (STE-137) transfected with rtERα (139). Similarly, the transactivation ability of another piscine ERα, the Oreochromis aureus (OaERα), also exhibits reduced activity at temperatures above its normal physiological range (141). The molecular basis for the reduced rtERα function at elevated temperature is unclear, though other fish proteins have been shown to exhibit optimal activity at lower temperatures than their mammalian counterparts (142-144). Interestingly, exchanging a 35 amino acid stretch, between the OaERα and the chicken ER (cER) partially rescued the thermal deficient transactivation of the OaERα (141), which suggests that additional factors also contribute to the temperature sensitive phenotype of piscine ERs.

Domain interchange and the generation of chimeric receptors revealed that the rtER (hERα C domain) chimeric receptor exhibited greater DNA binding affinity, while the rtER (hERα E domain) chimeric receptor displayed an increased E2 transactivational response compared to wild-type rtERα (145). These results suggest that some of the functional differences between hERα and rtERα reside within the DNA-binding domain. However, competitive binding studies using GST-hERα and GST-rtERα fusion proteins

Table 1. Comparison of the affinity for 17β-estradiol among different vertebrate ERs.

Vertebrate Species	Source	K_d in nM	Reference
Amphibians			
Urodele Amphibian	Testis	0.1	(146)
Xenopus	Liver cytosol	0.5	(147)
	Hepatocytes	0.4	(148)
Avian			
Chicken	Oviduct cytosol	0.23	(149)
	Oviduct cytosol	3.7	(150)
	Liver cytosol	0.7	(151)
Japanese quail	Liver cytosol	0.2	(152)
Fish			
Atlantic croaker	Testis	0.33-0.4	(153)
β	In vitro transcribed	1.38	(91)
γ	In vitro transcribed	1.16	(91)
Atlantic Salmon	Liver cytosolic	2-4	(154)
	Liver nuclear	5-6	(154)
Catfish	Liver cytosolic	1-1.3	(155)
Rainbow trout α	In vitro transcribed	5	(138)
Mammals			
Human α	Liver cytosol	0.47	(156)
	ER mammalian cells	0.2	(157)
	Full length in yeast	0.35	(158)
	Truncated in yeast	1.0	(158)
	ER–protein A fusion	1.49	(158)
Mouse	Uterine cytosol	1.4	(159)
α	In vitro transcribed	0.2	(160)
β	In vitro transcribed	0.5	(160)
Pig	Uterine cytosol	0.53	(149)
Rat	Uterine cytosol	0.7	(161)
α	Expressed in COS-1 cells	0.8	(161)
	In vitro transcribed	0.1	(162)
β	In vitro transcribed	0.4	(162)
Reptiles			
Alligator	Oviduct	0.5	(163)
Turtle	Liver - cytosolic	17	(164)
	Liver - nuclear	17	(164)
	Testis - cytosol	0.7	(165)

consisting of only the D, E and F domains have identified several differences in relative binding affinities of estrogenic chemicals, supporting the hypothesis that the LBD significantly contributes to the reported differences between hERα and rtERα (166,167) (Chapter 3 & 4).

Atrazine and simazine, two chloro-S-triazine derived pesticides were unable to induce expression of a hERα regulated reporter gene transiently transfected in MCF-7 cells (168), while atrazine and other chloro-S-triazines were able to compete with tritiated E2 for binding to the American alligator ER (163). The pig ER exhibits significantly greater affinity for α zearalenol, a metabolite of the mycotoxin zearalenone, than does the ER from the Leghorn chicken (149).

Genistein has been shown to bind with 30-fold greater affinity to hERβ than hERα (162). The recent report of the crystal structure of ERβ in complex with genistein has suggested that this ligand preference may be attributed to two conservative mutations: leucine 384 and methionine 421 in ERα are replaced by methionine 336 and isoleucine 373 in ERβ (74). Comparison of the amino acid differences within the ligand binding pockets of ER from several different species reveals that methionine 421 in hERα is replaced by phenylalanine 175 in the anolis ER and leucine 413 in xenopus ER, whereas leucine 349 and methionine 528 in hERα are substituted with methionine 317 and isoleucine 496 in the rtERα (Figure 12). Although, these residue differences are considered to be conservative substitutions, they affect the volume and hydrophobic character of the binding pocket (74,169). Taken together, these observations suggest that these residue substitutions may contribute to the observed differences in the relative binding affinities of select EEDs. Recent crystallography data has demonstrated that both

the volume and hydrophobic character of the binding pocket can influence ligand binding. For example, the volume of the probe-occupied ligand pocket of ERα-E2 crystal complex has been determined to be 490 Å while that of ERβ-genistein is 390 Å with the reduction being primarily due to the replacement of the leucine 384 in hERα with a bulkier methionine 336 residue in hERβ (74). This allows the residues that line the pocket to pack more tightly around genistein, stabilizing the ligand in the binding pocket in ERβ (74), and possibly resulting in greater binding affinity.

References

1. Human, P. (1994) *Human Pharmacology*, Second Edition Ed. (Brody, T. M., Larner, J., Minneman, K. P., and Neu, H. C., Eds.), Mosby, St. Louis

2. Razandi, M., Pedram, A., and Levin, E. R. (2000) *Mol Endocrinol* **14**(9), 1434-47.

3. Comp, E. (1987) *Fundamentals of comparative vertebrate endocrinology* (Chester-Jones, I., Ingelton, P. M., and Phillips, J. G., Eds.), Plenum Press, New York and London

4. Dutertre, M., and Smith, C. L. (2000) *J Pharmacol Exp Ther* **295**(2), 431-7.

5. Robertson, J. A., Zhang, Y., and Ing, N. H. (2001) *J Steroid Biochem Mol Biol* **77**(4-5), 281-7.

6. Grainger, D. J., and Metcalfe, J. C. (1996) *Nat Med* **2**(4), 381-5.

7. Grese, T. A., and Dodge, J. A. (1998) *Curr Pharm Des* **4**(1), 71-92.

8. Berry, M., Metzger, D., and Chambon, P. (1990) *EMBO J* **9**(9), 2811-2818.

9. McLachlan, J. A., and Korach, K. S. (1995) *Environ Health Perspect* **103 Suppl 7**, 3-4.

10. European Workshop on the Impact of Endocrine Disruptors on Human Health and Wildlife (1996) Vol. 1452, pp. 103-120., Weybridge, U.K.

11. Davis, D. L., Bradlow, H. L., Wolff, M., Woodruff, T., Hoel, D. G., and Anton-Culver, H. (1993) *Environ Health Perspect* **101**(5), 372-377.

12. Wolff, M. S., Toniolo, P.G., Lee, E.W., Rivera, M., Dubin, N. (1993) *J Natl Cancer Inst* **85**(8), 648-652.

13. Giwercman, A., Carlsen, E., Keiding, N., and Skakkebaek, N. E. (1993) *Environ Health Perspect* **101 Suppl 2**, 65-71.

14. Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1992) *Bmj* **305**(6854), 609-613.

15. Tilson, H. A., and Kodavanti, P. R. (1997) *Neurotoxicology* **18**(3), 727-743.

16. Colborn, T., vom Saal, F.S., Soto, A.M. (1993) *Environ. Health Perspect.* **101**, 378-384.

17. Guillette, L. J. J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F., Woodward, A.R. (1994) *Environ Health Perspect* **102**(8), 680-688.

18. EDSTAC, F. R. (1998), pp. 71541-71568, Federal Register Notice.

19. McLachlan, J. A. (1993) *Environ Health Perspect* **101**(5), 386-387.

20. Safe, S. H. (1995) *Environ. Health Perspect.* **103,** 346-351.

21. Harrison, P. T. C., Holmes, P. Humfrey, C.D.N. (1997) *Sci Total Environ* **205,** 97-106.

22. Katzenellenbogen, J. A. (1995) *Environ Health Perspect* **103 Suppl 7,** 99-101.

23. Carlsen, E., Giwercman, A., Keiding, N., Skakkebaek, N.E. (1995) *Environ Health Perspect* **103**(suppl 7), 137-139.

24. Sharpe, R. M., Skakkebaek, N.E. (1993) *Lancet* **341**(8857), 1392-1395
25. Irvine, D. S. (1994) *Br Med J* **309**(6952), 476.

26. Reijnders, P. J. (1986) *Nature* **324**(6096), 456-457.

27. Guillette, L. J. J., Pickford, D.B., Crain, D.A., Rooney, A.A., Percival, H.F. (1996) *Gen Comp Endocrinol* **101**(1), 32-42.

28. Fry, D. M. (1995) *Environ Health Perspect* **103**(suppl 7), 165-171.

29. O'Shea, T. J., Fleming, W.J., Cromartie, E. (1980) *Science* **209**(4455), 509-560.

30. Von Westernhagen, H., Rosenthal, H., Dethlefsen, V., Ernst, W., Harms, U.L., Hansen, P.D. (1981) *Aquatic Tox* **1,** 85-99.

31. Fry, D. M., Toone, C.K. (1981) *Science* **213,** 922-924.

32. Crews, D., Bergeron, J. M., and McLachlan, J. A. (1995) *Environ Health Perspect* **103**(Suppl 7), 73-77.

33. Crews, D., Bergeron, J.M., Bull, J.J., Flores, D., Tousignant, A., Skipper, J.K., Wibbels, T. (1994) *Dev Genet* **15**(3), 297-312.

34. Sumpter, J. P., and Jobling, S. (1995) *Environ Health Perspect* **103 Suppl 7,** 173-178.

35. Evans, R. (1988) *Science* **240,** 889-895.

36. Adlercreutz, H., Hockerstedt, K., Bannwart, C., Bloigu, S., Hamalainen, E., Fotsis, T., and Ollus, A. (1987) *J Steroid Biochem* **27**(4-6), 1135-1144.

37. Hodgert Jury, H., Zacharewski, T. R., and Hammond, G. L. (2000) *J Steroid Biochem Mol Biol* **75**(2-3), 167-176.

38. Chen, S., Kao, Y. C., and Laughton, C. A. (1997) *J Steroid Biochem Mol Biol* **61**(3-6), 107-115.

39. Kao, Y. C., Zhou, C., Sherman, M., Laughton, C. A., and Chen, S. (1998) *Environ Health Perspect* **106**(2), 85-92.

40. Loomis, A. K., and Thomas, P. (2000) *Biol Reprod* **62**(4), 995-1004.

41. Revelli, A., Massobrio, M., and Tesarik, J. (1998) *Endocr Rev* **19**(1), 3-17.

42. Blumberg, B., Sabbagh, W., Jr., Juguilon, H., Bolado, J., Jr., van Meter, C. M., Ong, E. S., and Evans, R. M. (1998) *Genes Dev* **12**(20), 3195-205.

43. Moore, J. T., and Kliewer, S. A. (2000) *Toxicology* **153**(1-3), 1-10

44. Htun, H., Holth, L. T., Walker, D., Davie, J. R., and Hager, G. L. (1999) *Mol Biol Cell* **10**(2), 471-486.

45. Hager, G. L., Lim, C. S., Elbi, C., and Baumann, C. T. (2000) *J Steroid Biochem Mol Biol* **74**(5), 249-254.

46. Mangelsdorf, D. J., Evans, R.M. (1995) *Cell* **83**(6), 841-850.

47. Enmark, E., and Gustafsson, J. A. (1996) *Mol Endocrinol* **10**(11), 1293-307.

48. Issemann, I., and Green, S. (1990) *Nature* **347**(6294), 645-650.

49. Wang, L. H., Tsai, S. Y., Cook, R. G., Beattie, W. G., Tsai, M. J., and O'Malley, B. W. (1989) *Nature* **340**(6229), 163-166.

50. Sladek, F. M., Zhong, W. M., Lai, E., and Darnell, J. E., Jr. (1990) *Genes Dev* **4**(12B), 2353-2365.

51. Giguere, V. (1999) *Endocr Rev* **20**(5), 689-725.

52. Giguere, V., Yang, N., Segui, P., and Evans, R. M. (1988) *Nature* **331**(6151), 91-94.

53. Song, C., Kokontis, J. M., Hiipakka, R. A., and Liao, S. (1994) *Proc Natl Acad Sci U S A* **91**(23), 10809-10813.

54. Lala, D. S., Rice, D. A., and Parker, K. L. (1992) *Mol Endocrinol* **6**(8), 1249-1258.

55. Zanaria, E., Muscatelli, F., Bardoni, B., Strom, T. M., Guioli, S., Guo, W., Lalli, E., Moser, C., Walker, A. P., McCabe, E. R., and et al. (1994) *Nature* **372**(6507), 635-641.

56. Seol, W., Choi, H. S., and Moore, D. D. (1996) *Science* **272**(5266), 1336-1339.

57. Johansson, L., Thomsen, J. S., Damdimopoulos, A. E., Spyrou, G., Gustafsson, J. A., and Treuter, E. (1999) *J Biol Chem* **274**(1), 345-353.

58. Mader, S., Kumar, V., de Verneuil, H., and Chambon, P. (1989) *Nature* **338**(6212), 271-274.

59. Umesono, K., and Evans, R. M. (1989) *Cell* **57**(7), 1139-1146.

60. Kumar, V., and Chambon, P. (1988) *Cell* **55**(1), 145-156.

61. Dahlman-Wright, K., Wright, A., Gustafsson, J. A., and Carlstedt-Duke, J. (1991) *J Biol Chem* **266**(5), 3107-3112.

62. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995) *Nature* **377**(6548), 397-404.

63. Chen, J. D., and Evans, R. M. (1995) *Nature* **377**(6548), 454-457.

64. Wang, C., Fu, M., Angeletti, R. H., Siconolfi-Baez, L., Reutens, A. T., Albanese, C., Lisanti, M. P., Katzenellenbogen, B. S., Kato, S., Hopp, T., Fuqua, S. A., Lopez, G. N., Kushner, P. J., and Pestell, R. G. (2001) *J Biol Chem* **276**(21), 18375-18383.

65. Metzger, D., Losson, R., Bornert, J. M., Lemoine, Y., and Chambon, P. (1992) *Nucleic Acids Res* **20**(11), 2813-2817.

66. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) *Cell* **59**, 477-487.

67. Montano, M. M., Muller, V., Trobaugh, A., and Katzenellenbogen, B. S. (1995) *Molecular Endocrinology* **9**(7), 814-825.

68. Warnmark, A., Almlof, T., Leers, J., Gustafsson, J. A., and Treuter, E. (2001) *J Biol Chem* **276**, 23397-23404.

69. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375**(6530), 377-382.

70. Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **378**(6558), 681-689.

71. Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995) *Nature* **378**(6558), 690-697.

72. Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D., and Gronemeyer, H. (1996) *Nat Struct Biol* **3**(1), 87-94.

73. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**(6652), 753-758.

74. Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J., and Carlquist, M. (1999) *Embo J* **18**(17), 4608-4618.

75. Matias, P. M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Joschko, S., Scholz, P., Wegg, A., Basler, S., Schafer, M., Egner, U., and Carrondo, M. A. (2000) *J Biol Chem* **275**(34), 26164-26171.

76. Sack, J. S., Kish, K. F., Wang, C., Attar, R. M., Kiefer, S. E., An, Y., Wu, G. Y., Scheffler, J. E., Salvati, M. E., Krystek, S. R., Jr., Weinmann, R., and Einspahr, H. M. (2001) *Proc Natl Acad Sci U S A* **98**(9), 4904-4909.

77. Gee, A. C., and Katzenellenbogen, J. A. (2001) *Mol Endocrinol* **15**(3), 421-428.

78. Egea, P. F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., and Moras, D. (2000) *Embo J* **19**(11), 2592-25601.

79. Mosselman, S., Polman, J., and Dijkema, R. (1996) *FEBS Lett* **392**(1), 49-53.

80. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J.A. (1996) *Proc Natl Acad Sci U S A* **93**(12), 5925-5930.

81. Giguere, V., Tremblay, A., and Tremblay, G. B. (1998) *Steroids* **63**(5-6), 335-339.

82. Cowley, S., Hoare, S., Mosselman, S., and Parker, M. (1997) *J Biol Chem* **272**(32), 19858-19862.

83. Pettersson, K., Grandien, K., Kuiper, G. G., and Gustafsson, J. A. (1997) *Mol Endocrinol* **11**(10), 1486-1496.

84. Pace, P., Taylor, J., Suntharalingam, S., Coombes, R. C., and Ali, S. (1997) *J Biol Chem* **272**(41), 25832-25838.

85. Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., Sar, M., Korach, K. S., Gustafsson, J. A., and Smithies, O. (1998) *Proc Natl Acad Sci U S A* **95**(26), 15677-15682.

86. Couse, J. F., Hewitt, S. C., Bunch, D. O., Sar, M., Walker, V. R., Davis, B. J., and Korach, K. S. (1999) *Science* **286**(5448), 2328-2331.

87. Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., and Smithies, O. (1993) *Proc Natl Acad Sci U S A* **90**(23), 11162-11166.

88. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. (1997) *Science* **277**(5331), 1508-1510.

89. Webb, P., Nguyen, P., Valentine, C., Lopez, G. N., Kwok, G. R., McInerney, E., Katzenellenbogen, B. S., Enmark, E., Gustafsson, J. A., Nilsson, S., and Kushner, P. J. (1999) *Mol Endocrinol* **13**(10), 1672-1685.

90. Zou, A., Marschke, K. B., Arnold, K. E., Berger, E. M., Fitzgerald, P., Mais, D. E., and Allegretto, E. A. (1999) *Mol Endocrinol* **13**(3), 418-430.

91. Hawkins, M. B., Thornton, J. W., Crews, D., Skipper, J. K., Dotte, A., and Thomas, P. (2000) *Proc Natl Acad Sci U S A* **97**(20), 10751-10756.

92. Robinson-Rechavi M, Marchand O, Escriva H, Bardet PL, Zelus D, Hughes S, and V, L. (2001) *Genome Res* **11**(5), 781-788.

93. Shiau, A. K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., Greene, G.L. (1998) *Cell* **95**(7), 927-937.

94. Pike, A. C., Brzozowski, A. M., Walton, J., Hubbard, R. E., Thorsell, A., Li, Y., Gustafsson, J., and Carlquist, M. (2001) *Structure* **9**(2), 145-153.

95. Ali, S., Metzger, D., Bornert, J. M., and Chambon, P. (1993) *Embo J* **12**(3), 1153-1160.

96. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., and et al. (1995) *Science* **270**(5241), 1491-1494.

97. White, R., Sjoberg, M., Kalkhoven, E., and Parker, M. G. (1997) *Embo J* **16**(6), 1427-1435.

98. Tanenbaum, D. M. (1998) in *Graduate School* Vol. 1, pp. 149, Yale univeristy.

99. Glass, C. K., and Rosenfeld, M. G. (2000) *Genes Dev* **14**(2), 121-141.

100. Onate, S. A., Tsai, S.Y., Tsai, M.J., O'Malley, B.W. (1995) *Science 1995* **270**(5240), 1354-1357.

101. Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., Brown, M. (1994) *Science* **264**(5164), 1455-1458.

102. Hong, H., Kohli, K., Garabedian, M., and Stallcup, M. (1997) *Mol Cell Biol* **17**(5), 2735-2744.

103. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) *EMBO J.* **1996,** 3667-3675.

104. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) *Science* **277**(5328), 965-968.

105. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90**(3), 569-580.

106. Takeshita, A., Cardona, G. R., Koibuchi, N., Suen, C. S., and Chin, W. W. (1997) *J Biol Chem* **272**(44), 27629-27634.

107. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**(6634), 677-684.

108. Li, H., Gomes, P. J., and Chen, J. D. (1997) *Proc Natl Acad Sci U S A* **94**(16), 8479-8484.

109. Hankinson, O. (1995) *Annu Rev Pharmacol Toxicol* **35,** 307-340.

110. Gu, Y. Z., Hogenesch, J. B., and Bradfield, C. A. (2000) *Annu Rev Pharmacol Toxicol* **40,** 519-561.

111. Heery, D. M., Kalkhoven, E., Hoare, S., Parker, M.G. (1997) *Nature* **387**(6634), 733-736.

112. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) *Genes Dev* **12**(21), 3343-3356.

113. Zhou, G., Cummings, R., Li, Y., Mitra, S., Wilkinson, H. A., Elbrecht, A., Hermes, J. D., Schaeffer, J. M., Smith, R. G., and Moller, D. E. (1998) *Mol Endocrinol* **12**(10), 1594-1604.

114. Bramlett, K. S., Wu, Y., and Burris, T. P. (2001) *Mol Endocrinol* **15**(6), 909-922.

115. Routledge, E. J., White, R., Parker, M. G., and Sumpter, J. P. (2000) *J Biol Chem* **275**(46), 35986-35993.

116. Lee, S. K., Anzick, S. L., Choi, J. E., Bubendorf, L., Guan, X. Y., Jung, Y. K., Kallioniemi, O. P., Kononen, J., Trent, J. M., Azorsa, D., Jhun, B. H., Cheong, J. H., Lee, Y. C., Meltzer, P. S., and Lee, J. W. (1999) *J Biol Chem* **274**(48), 34283-34293.

117. Tai, H., Kubota, N., and Kato, S. (2000) *Biochem Biophys Res Commun* **267**(1), 311-316.

118. Xu, J., Qiu, Y., DeMayo, F. J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1998) *Science* **279**(5358), 1922-1925.

119. Xu, J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C., and O'Malley, B. W. (2000) *Proc Natl Acad Sci U S A* **97**(12), 6379-6384.

120. Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Nature* **389**(6647), 194-198.

121. Baumann, C. T., Maruvada, P., Hager, G. L., and Yen, P. M. (2001) *J Biol Chem* **276**(14), 11237-11245.

122. Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997) *Nature* **387**(6628), 49-55.

123. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**(6628), 43-48.

124. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000) *Cell* **103**(6), 843-852.

125. Rachez, C., Gamble, M., Chang, C. P., Atkins, G. B., Lazar, M. A., and Freedman, L. P. (2000) *Mol Cell Biol* **20**(8), 2718-2726.

126. Walker, P., Germond, J. E., Brown-Luedi, M., Givel, F., and Wahli, W. (1984) *Nucleic Acids Res* **12**(22), 8611-8626.

127. Klein-Hitpass, L., Schorpp, M., Wagner, U., and Ryffel, G. U. (1986) *Cell* **46**(7), 1053-1061.

128. Driscoll, M. D., Sathya, G., Muyan, M., Klinge, C. M., Hilf, R., and Bambara, R. A. (1998) *J Biol Chem* **273**(45), 29321-29330.
129. Hyder, S., Nawaz, Z., Chiappetta, C., Yokoyama, K., and Stancel, G. (1995) *J Biol Chem* **270**(15), 8506-8513.
130. Richard, S., and Zingg, H. H. (1990) *J Biol Chem* **265**(11), 6098-6103.
131. Martinez, E., Givel, F., and Wahli, W. (1987) *Embo J* **6**(12), 3719-3727.
132. Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990) *Embo J* **9**(5), 1603-1614.
133. Weisz, A., and Rosales, R. (1990) *Nucleic Acids Res* **18**(17), 5097-5106.
134. Berry, M., Nunez, A. M., and Chambon, P. (1989) *Proc Natl Acad Sci U S A* **86**(4), 1218-1222.
135. Kato, S., Tora, L., Yamauchi, J., Masushige, S., Bellard, M., and Chambon, P. (1992) *Cell* **68**(4), 731-742.
136. Lee, M. O., Liu, Y., and Zhang, X. K. (1995) *Mol Cell Biol* **15**(8), 4194-4207.
137. Klinge, C. M., Bodenner, D. L., Desai, D., Niles, R. M., and Traish, A. M. (1997) *Nucleic Acids Res* **25**(10), 1903-1912.
138. Pakdel, F., Le Gac, F., Le Goff, P., and Valotaire, Y. (1990) *Mol Cell Endocrinol* **71**(3), 195-204.
139. Le Drean, Y., Kern, L., Pakdel, F., and Valotaire, Y. (1995) *Mol. Cell. Endocrinol.* **109**, 27-35.
140. Petit, F., Valotaire, Y., and Pakdel, F. (1995) *Eur J Biochem* **233**(2), 584-592.
141. Tan, N. S., Frecer, V., Lam, T. J., and Ding, J. L. (1999) *Biochim Biophys Acta* **1452**(2), 103-120.
142. Klaunig, J. E., Ruch, R. J., and Goldblatt, P. J. (1985) *In Vitro Cell Dev Biol* **21**(4), 221-228.
143. de Toledo, F. G., Albuquerque, M. C., Goulart, B. H., and Chini, E. N. (1995) *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **111**(1), 93-98.
144. Pohl, R. J., Bend, J. R., Guarino, A. M., and Fouts, J. R. (1974) *Drug Metab Dispos* **2**(6), 545-555.

145. Petit, F. G., Valotaire, Y., and Pakdel, F. (2000) *Nucleic Acids Res* **28**(14), 2634-2642.

146. Mak, P., Callard, I.P., Callard, G.V. (1983) *Biol Reprod* **28**(2), 261-270.

147. Westley, B., Knowland, J. (1978) *Cell* **15**(2), 367-374.

148. Perlman, A. J., Wolffe, A.P., Champion, J., Tata, J.R. (1984) *Mol Cell Endocrinol* **38**(2-3), 151-161.

149. Fitzpatrik, D. W., Picken, C. A., Murphy, L. C., and Buhr, M. M. (1989) *Comp Biochem Physiol* **94C**(2), 691-694.

150. Mulvihill, E. R., Palmiter, R.D. (1977) *J Biol Chem* **252**(6), 2060-2068.

151. Lazier, C. B. (1987) *J Steroid Biochem* **27**(4-6), 877-882.

152. Turner, R. T. (1984) *Gen Comp Endocrinol* **54**(3), 457-461.

153. Loomis, A. K., and Thomas, P. (1999) *Biol Reprod* **61**(1), 51-60.

154. Lazier, C. B., Lonergan, K., Mommsen, T.P. (1985) *Gen Comp Endocrinol* **57**(2), 234-245.

155. Nimrod, A. C., and Benson, W. H. (1997) *Toxicol Appl Pharmacol* **147**(2), 381-390.

156. Rossini, G. P., Baldini, G.M., Villa, E., Manenti, F. (1989) *Gastroenterology* **96**(4), 1102-1109.

157. Eriksson, H. A. (1982) *J Steroid Biochem* **17**(5), 471-477.

158. Wooge, C., H,. Nilsson, G,M,. Heierson, A., McDonnell, D.P., Katzenellenbogen, B.S. (1992) *Mol Endocrinol* **6**(6), 861-869.

159. Horigome, T., Golding, T.S., Quarmby, V.E., Lubahn, D.B., McCarty, K. Sr., Korach, K.S. (1987) *Endocrinology* **121**(6), 2099-2111.

160. Tremblay, G. B., Tremblay, A., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Labrie, F., Giguere, V. (1997) *Mol Endocrinol* **11**(3), 353-365.

161. Koike, S. S., M. Muramatsum, M. (1987) *Nucleic Acids Res* **15**(6), 2499-2513.

162. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., Gustafsson, J.A. (1997) *Endocrinology* **138**(3), 863-870.

163. Vonier, P. M., Guillette, J., L.J., McLachlan, J. A., and Arnold, S. F. (1997) *Biochem Biophys Res Comm* **232**(2), 308-312.

164. Ho, S. M., Fehrer, S., Yu, M., Liang, L.C., Press, D. (1988) *Gen Comp Endocrinol* **70**(3), 382-394.

165. Mak, P., Ho, S.M., Callard, I.P. (1983) *Gen Comp Endocrinol* **52**(2), 182-189.

166. Matthews, J. B., Celius, T., Halgren, R., and Zacharewski, T. R. (2000) *J Steroid Biochem Mol Bio* **74**(4), 223-234.

167. Matthews, J. B., and Zacharewski, T. R. (2000) *Toxicol Sci* **53**(2), 326-339.

168. Connor, K., Howell, J., Chen, I., Liu, H., Berhane, K., Sciarretta, C., Safe, S., and Zacharewski, T. (1995) *Fund. Appl. Toxicol.* **30**, 93-101.

169. Stickle, D. F., Presta, L. G., Dill, K. A., and Rose, G. D. (1992) *J Mol Biol* **226**(4), 1143-1159.

CHAPTER 3

Differential binding affinities of PCBs, HO-PCBs and aroclors with recombinant human, rainbow trout (Oncorhynchus mykiss) and green anole (Anolis carolinensis) estrogen receptors using a semi-high throughput competitive binding assay

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Differential Binding Affinities of PCBs, HO-PCBs, and Aroclors with Recombinant Human, Rainbow Trout *(Onchorhynkiss mykiss)*, and Green Anole *(Anolis carolinensis)* Estrogen Receptors, Using a Semi-High Throughput Competitive Binding Assay

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A comparative study was undertaken to assess the ability of 44 polychlorinated biphenyls (PCBs), 9 hydroxylated PCBs (HO-PCBs), and 8 aroclors at concentrations ranging from 1 nM to 10 μM to compete with [^{3}H]17β-estradiol (E2) for binding to bacterially expressed fusion proteins using a semi-high throughput competitive-binding assay. The fusion proteins consisted of the D, E, and F domains of human (α), cloned reptilian (*Anolis carolinensis*) and recloned rainbow trout (*Onchorhynkiss mykiss*) estrogen receptors (ER) linked to the glutathione *S*-transferase (GST) protein. GST-hERαdef (human), GST-aERdef (reptile) and GST-rtERdef (rainbow trout) fusion proteins exhibited high affinity for E2 with dissociation constants (K_d) of 0.4 ± 0.1 nM, 0.7 ± 0.2 nM, and 0.6 ± 0.1 nM, respectively. Of the 44 PCBs examined, only PCBs 104, 184, and 188 effectively competed with [^{3}H]E2 for binding to the GST-rtERdef protein with IC_{50} values ranging from 0.4–1.3 μM. In contrast, these same congeners only caused a 30% displacement of [^{3}H]E2 in GST-hERαdef and GST-aERdef proteins. Several additional congeners were found to bind to the GST-rtERdef fusion protein, although the degree of interaction varied among congeners. Among the HO-PCBs, 2′,3′,4′,5′-tetrachloro-4-biphenylol and 2,6,2′,6′-tetrachloro-4-biphenylol bound to all three fusion proteins with IC_{50} values ranging from 0.1–0.3 μM. Dimethyl sulphoxide (DMSO) concentrations of 20% significantly increased the ability of PCBs 104, 184, and 188 to compete with [^{3}H]E2 for binding to the GST-ERdef fusion proteins, whereas at 20% DMSO, a significant reduction in saturable binding was observed. These results demonstrate that ERs from different species exhibit differential ligand preferences and relative binding affinities for PCBs, which can be dramatically affected by DMSO concentration.

Key Words: **polychlorinated biphenyls; estrogenic endocrine disruptors; estrogen receptor; comparative; competitive binding.**

In recent years, there has been heightened concern regarding exposure to chemicals in the environment that may interfere with the endocrine system and adversely affect normal reproductive development and fitness of humans and wildlife (McLachlan and Korach, 1995). These substances, known as endocrine disrupting chemicals (EDCs), are commonly used throughout modern society. In response, the Safe Drinking Water Act and the Food Quality Protection Act were amended to require the United States Environmental Protection Agency (U.S. EPA) to screen and test for chemicals that mimic or inhibit the activities of estrogen, androgen, and thyroid hormones. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was established and charged with the development and implementation of a screening and testing strategy (EDSTAC, 1998). EDSTAC deliberations resulted in a tiered strategy consisting of prioritization followed by screening and testing approaches that involve a combination of *in vitro* and *in vivo* assays. The U.S. EPA is currently exploring the possibility of using high-throughput receptor binding and reporter gene assays to assist in prioritizing chemicals that require testing.

Much attention has been focused on estrogenic endocrine disruptors (EEDs). These chemicals encompass a wide range of substances including natural products, environmental pollutants, pharmaceuticals, and industrial chemicals (Colborn, 1993; Katzenellenbogen, 1995). Many of these chemicals do not share any obvious structural similarity to the endogenous ligand for the estrogen receptor (ER), 17β-estradiol (E2), which makes identification based solely on molecular structure difficult (Katzenellenbogen, 1995). It has been hypothesized that many of the effects elicited by estrogenic substances are the result of ER-mediated modulation of gene expression (McLachlan, 1993), although additional modes of action cannot be discounted.

The ER is a member of the nuclear receptor superfamily, a family of nuclear proteins that function as transcription factors to modulate gene expression in a ligand-dependent manner (Evans, 1988). As with other members of this family, the ER has a modular structure consisting of six domains (A–F) (Evans, 1988; Tenbaum and Baniahmad, 1997). The highly

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conserved DNA-binding domain (C domain) separates a highly variable NH_2-terminal region (A/B domains) and a COOH-terminal ligand-binding domain (D, E, and F domains). The ER and other steroid hormone receptors are activated by interaction with specific ligands that bind with high affinity to the ligand-binding domain. Ligand-occupied ERs undergo homodimerization, and the resulting complex binds to its cognate DNA target site. These sites are referred to as estrogen responsive elements (EREs) and are located in the regulatory region of estrogen-inducible genes. Once bound to the ERE, the ER-homodimer complex may induce or inhibit gene transcription, thereby altering the levels of proteins that are important for development, cell proliferation, and the maintenance of homeostasis. Consequently, the ER acts as the primary gatekeeper for initiation of a number of estrogenic responses.

Even though the physiological actions of the ER are conserved among different species, amino acid sequences of ligand binding domains are variable. This suggests that species may exhibit different responses and sensitivities to EEDs, and that one species may not be an appropriate surrogate for use in identifying and predicting responses in other species. A number of competitive-binding studies have shown that EEDs exhibit differential binding preferences and relative binding affinities for the ER of different species (Connor *et al.*, 1997; Fitzpatrik *et al.*, 1989; Le Drean *et al.*, 1995; Vonier *et al.*, 1997).

Polychlorinated biphenyls (PCBs) are a class of halogenated aromatic industrial compounds that are ubiquitous, persistent environmental contaminants detected in almost every ecosystem (Bellschmiter *et al.*, 1981). They were commercially manufactured as mixtures (e.g. Aroclors) containing varying degrees of chlorination made up of 140–150 of the 209 possible congeners (Mullin *et al.*, 1984; Safe, 1993; Schulz *et al.*, 1989). PCBs evoke or elicit a number of *in vitro* and *in vivo* responses. PCBs, and hydroxylated (HO)-PCBs have been identified in wildlife and humans, and they represent a class of EEDs that differs significantly from the endogenous ER ligand, E2. Coplanar congeners and their planar, mono-*ortho* substituted derivatives induce responses that correlate with their binding affinity for the aryl hydrocarbon receptor (AhR) and evoke 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-like responses (Safe, 1993). Non-coplanar (di-, tri- and tetra-*ortho* substituted) PCBs and some HO-PCBs induce multiple responses (Narasimhan *et al.*, 1991; Schuur *et al.*, 1998; Van den Berg *et al.*, 1991) including *in vitro* and *in vivo* estrogenic activities independent of the AhR (Bitman and Cecil, 1970; Fielden *et al.*, 1997; Li *et al.*, 1994).

In order to investigate the ability of PCBs to compete with E2 for binding to the ER and to identify potential differences in ER binding among species, a comparative study was undertaken in which a semi-high throughput competitive-binding assay, using bacterially expressed GST-ER fusion proteins, was developed. In this study, 44 PCB congeners, 8 commercial Aroclor mixtures, and 9 HO-PCBs, 7 of which have been detected in human serum (Bergman *et al.*, 1994; Moore *et al.*, 1997) were examined for their ability to compete with [^{3}H]E2 for binding to the recombinant ERs from human, green anole (*Anolis carolinensis*) and rainbow trout (*Onchorhynkiss mykiss*).

MATERIALS AND METHODS

Chemicals. Polychlorinated biphenyls (PCB) International Union of Pure and Applied Chemistry (IUPAC) # 41, 51, 58, 60, 68, 78, 91, 99, 104, 112, 115, 126, 143, 153, 169, 173, 184, 188, 190, and 193 were purchased from AccuStandard (New Haven, CT) and provided by M. Tysklind (Umeå University, Umeå, Sweden). PCBs IUPAC # 18, 44, 45, 47, 54, 70, 74, 84, 87, 101, 128, 138, 151, 158, 168, 177, 178, 183, 187, and 194; Aroclors 1016, 1221, 1232, 1242, 1248, 1254, 1260, and 1268 were synthesized or provided by S. Safe (Texas A&M University, College Station, TX). Hydroxylated PCBs 2,3,5,3′,4′-pentachloro-4-biphenylol; 2,3,5,2′,4′,5′-hexachloro-4-biphenylol; 2,4,5,2′,3′,4′,5′′-heptachloro-3-biphenylol; 3,5,2′,3′,4′-pentachloro-4-biphenylol; 2,3,5,2′,3′,4′-hexachloro-4-biphenylol; 2,3,5,2′,3′,4′,5′-heptachloro-4-biphenylol; 2,3,5,6,2′,4′,5′,-heptachloro-4-biphenylol; 2,6,2′,6′-tetrachloro-4-biphenylol; and 2′,3′,4′,5′-tetrachloro-4-biphenylol were synthesized by S. Safe (Connor *et al.*, 1997; Safe *et al.*, 1995). Dimethyl sulphoxide (DMSO) and 17β-estradiol (E2) were obtained from Sigma (St. Louis, MO). [2,4,6,7,16,17-^{3}H] 17β-estradiol ([^{3}H]E2; 123 Ci/mmol) was purchased from New England Nuclear (Boston, MA). MicroScint 20 was obtained from Packard Instruments (Meriden, CT). Isopropylthio-β-D-galactoside (IPTG) and bovine serum albumin (BSA) were obtained from Fisher Scientific (Pittsburgh, PA). The glutathione *S*-transferase (GST) expression vector, pGEX6p3, glutathione (GSH) sepharose and XK16 columns were purchased from Amersham/Pharmacia (Piscataway, NJ). SuperScript II reverse transcriptase and Trizol Reagent were purchased from Life Technologies (Gaithesburg, MD). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA), and restriction enzymes and Taq DNA polymerase were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). All other chemicals and biochemicals were of the highest quality available from commercial sources.

RNA isolation. Total RNA from a 1 cm^3 liver section from a female green anole (*Anolis carolinensis*; kindly provided by J. Wade, Departments of Psychology and Zoology, Michigan State University) was isolated using Trizol Reagent. The Trizol Reagent procedure is a modification of the single step-RNA isolation method developed by Chomczynski and Sacchi, (1987). Green anole liver sections were homogenized in the presence of Trizol Reagent, using a Brinkman Polytron homogenizer. Following a 5-min incubation at ambient temperature, chloroform was added and the mixture was separated by centrifugation at 12,000 × *g* for 15 min at 4°C. The aqueous layer containing the isolated RNA was removed and the RNA was precipitated using isopropanol. The RNA was pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C, and the resulting pellet was washed with 75% ethanol diluted with diethyl pyrocarbonate (DEPC)-treated sterile water. The pellet was then air dried and resuspended in DEPC-treated sterile water. RNA was stored at −80°C until use.

Cloning of green anole estrogen receptor DEF domain. Total RNA (5 μg) was incubated for 10 min at 70°C with 500 nM oligo dT primer (PR1r). Following a 5 min incubation on ice, the mRNA was reverse transcribed in a 20 μl reaction mixture containing PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), 10 mM dithiothreitol (DTT), 500 μM dNTPs and 200 units of SuperScript II reverse transcriptase at 42°C for 50 min. The reaction was terminated with a 15 min incubation at 70°C. The reverse transcription (RT) reaction was then incubated with 1 unit of RNase H for 30 min at 37°C. One tenth of the RT reaction was used in the subsequent PCR reactions.

RACE (rapid amplification of cDNA ends) PCR reactions were performed according to the manufacturer's instructions (Life Technologies). Three de-

TABLE 1

PCR Primers Used in the Cloning and Recloning of the Green Anole and Rainbow Trout Estrogen Receptor (ER) D, E and F Domains and in the Construction of the GST-ERdef Containing Plasmids

Primer	Sequence	Restriction enzyme	Description
PR1r[a]	5'-GGCCACGCGTCGACTAGTACT$_{17}$-3'	SalI[b]	OligodT primer[c]
PR2r	5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3'	SalI	Universal amplification primer[c]
PR3f[d]	5'-TA/C/TCAAGGA/G/TCAC/TAAC/TGACTAC/TATGTGC/TC-3'	-	Degenerate primer based on ER DNA binding domain (human ERα a.a. 213–222)
PR4f	5' AAAAGAATTCGGATCCGC/TTAC/TGAAGTA/GGGA/C/ GATGATGAAAGG-3'	EcoRI BamHI	Degenerate primer based on ER DNA binding domain, upstream of PR3f (human ERα a.a. 244–253)
PR5r	5'-GCGGCCGCCTCGAGGA/G/TGAA/GAGA/GATGAGGA-GGAGGAGCT-3'	NotI XhoI	Degenerate primer based on ER hormone binding domain (human ERα a.a. 517–506)
PR6f	5'-CAAAGAATTCGGATCCCATGTTGCTGGCCGCTTCTTCTC-3'	EcoRI BamHI	Anole ER specific primer
PR7f	5'-AAAAGGATCCATGTTGAAACACAAGCGCCAGAGAG-3'	BamHI	Human ERα DEF forward primer (pGEX)
PR8r	5'-AAAACTCGAGTCAGACTGTGGCAGGGAAACCCT-3'	XhoI	Human ERα DEF reverse primer (pGEX)
PR9f	5'-AAAAGGATCCATGCTGAAACACAAACGTCAAAGAG-3'	BamHI	Anole ER DEF forward primer (pGEX)
PR10r	5'-AAAAGGATCCCTCGAGTCAAATTGCTTCCTGCTCAT-TTCCC-3'	BamHI XhoI	Anole ER DEF reverse primer (pGEX)
PR11f	5'-AAAAGGATCCCTCGAGGGCGGGTTCTCAGGGATAAGCG-3'	XhoI	Rainbow trout ER DEF reverse primer (pGEX)
PR12r	5'-AAAACTCGAGTCACGGAATGGGCATCTGGTCTG-3'	XhoI	Rainbow trout ER DEF reverse primer (pGEX)

[a] "r" denotes reverse primer.
[b] Restriction enzyme sites are underlined.
[c] Commercially available primers provided in the 3'RACE kit (Life Technologies).
[d] "f" denotes forward primer.

generate primers were used in the cloning strategy. The oligonucleotides were identified from a consensus sequence derived by a multiple sequence alignment of the ERs from 10 different species. Two of the primers (PR3f and PR4f; see Table 1) were based on the highly conserved ER DNA binding domain and the third primer, PR5r, was derived from a highly conserved region in the ligand binding domain (LBD). Optimal PCR reaction conditions were determined to be 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 nM of each primer, 200 μM dNTPs and 2.5 units of Taq polymerase. Following the addition of template, the samples were incubated at 95°C for 2 min and amplified for 35 cycles. Each cycle included: 1 min denaturation at 95°C, 1 min annealing at 62°C and 2 min elongation at 72°C. Ten percent of the first strand synthesis reaction was PCR amplified using primers PR3f and PR2r. Using a nested PCR strategy, a 2 μl aliquot of the initial PCR reaction was used as a template for a subsequent PCR amplification using primers PR4f and PR2r. The products from the second round of PCR were used as a template in a third PCR reaction using primers PR4f and PR5r. This reaction produced a fragment of approximately 800 bp, which was digested with BamHI and XhoI, cloned into the eukaryotic expression vector, pTL1 and sequenced using ABI/Prism automated sequencing (Perkin Elmer Applied Biosystems; Foster City, CA). Based on this sequence, a green anole ER-specific primer (PR6f) was designed and used with PR2r to amplify the 3' end of the ER using a 2 μl aliquot of the PR4f/PR2r PCR reaction as template. The resulting 1100-bp product was cloned into pGEM plasmid (Promega) and sequenced using ABI/Prism automated sequencing. The boundaries of the green anole ER D, E and F domains were determined by sequence alignment to the human ERα. Sequence analysis was performed using MacVector 6.5 and the GCG Wisconsin Package (Oxford Molecular Ltd., Beaverton OR).

Recloning of rainbow trout ER DEF domains. Total RNA (1 μg) from the liver of a female rainbow trout (*Onchorhynkus mykiss*; kindly provided by S. Wagner, Department of Physiology, University of Western Ontario) was reverse transcribed as previously described (Gillesby and Zacharewski, 1999) in the presence of first-strand buffer (50 mM Tris–HCl (pH 8.3), 37.5 mM KCl, 1.5 mM $MgCl_2$), 10 mM DTT, 500 nM reverse primer (PR14r), 1 mM dNTPs, and 40 units of SuperScript II. The RT reaction was incubated for 10 min at 25°C, followed by 50 min at 42°C, 15 min at 70°C and 5 min at 4°C. The entire cDNA product produced in the RT reaction was used in subsequent PCR reactions. The PCR reaction mixture, containing Thermopol buffer (20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Triton X-100), 200 μM dNTPs, 1 mM $MgSO_4$, 2 μM primers (PR13f/PR14r), and 1.25 units of Vent DNA polymerase was amplified for 30 cycles using the following conditions: 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min.

Construction of GST-ERdef fusion proteins. The plasmids pGEXhERαdef, pGEXaERdef, and pGEXrtERdef were constructed by PCR amplification of the human ERα (kindly provided by P. Chambon, INSERM U184, Strasbourg, France), green anole, and rainbow trout ER DEF domains using primers PR7f/PR8r, PR9f/PR10r, and PR11f/PR12r, respectively. The fragments were digested with the appropriate restriction enzymes (see Table 1)

and ligated into the GST fusion protein expression vector, pGEX6p3. The PCR amplification was performed using Vent DNA polymerase (New England Biolabs) as described above. The PCR reaction mixture containing Thermopol buffer, 200 μM dNTPs, 1 mM $MgSO_4$, 500 nM primer, and 1.25 units of polymerase was heated to 94°C for 5 min followed by 35 rounds of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min 45 s. The sequence of each construct was confirmed with restriction enzyme digest and ABI/Prism automated sequencing.

Expression and purification of GST ER fusion proteins. Overnight cultures of *E. coli* strain BL21 (Amersham/Pharmacia) containing pGEX-ERdef constructs were diluted 1:100 in 500 ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, pH 7.5) containing 100 μg ampicillin/ml and incubated at 37°C with constant shaking. The cells were grown to an optical density of approximately 1.0 at 600 nm, and induced with IPTG at a final concentration of 1 mM. The induced cultures were incubated for 4 h at 37°C, then pelleted by centrifugation at 1000 × *g* for 10 min at 4°C. Cell pellets were resuspended in 25 ml of buffer A (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 50 mM NaCl, and 10% (v/v) glycerol, pH 7.5) containing 0.1 mg/ml lysozyme, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin,and 10 μg/ml pepstatin A. Cells were then lysed by sonication on ice for 3 × 15 s, separated by 10 s intervals. Tween20, to a final concentration of 0.1%, was added to the cellular debris and incubated for 30 min at 4°C under constant shaking. Cell debris was pelleted by centrifugation at 20,000 × *g* for 40 min at 4°C. Supernatants were stored at −80°C until further use.

The supernatants containing the GST fusion proteins were applied to an XK16 column containing GSH sepharose pre-equilibrated with buffer A at a constant flow rate of 0.5 ml/min at 4°C. After adsorption of the protein, the GSH sepharose was washed with 100 ml of buffer B (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 150 mM NaCl and 10% (v/v) glycerol, pH 7.5). Bound proteins were eluted in 25 ml of buffer C (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 150 mM NaCl and 10% (v/v) glycerol, pH 8.0) containing 10 mM GSH. The partially purified protein was concentrated to a 1-ml final volume using Millipore Ultrafree-15 filter columns with a 50-kDa molecular weight cutoff (Millipore Corp., Bedford MA). Protein concentration was determined using the Bradford (1976) method. Protein was diluted to 0.5 mg/ml and stored at −80°C until further use. Partially purified fusion proteins were separated by SDS-PAGE according to Laemmli (1970), using a 4% stacking and 10% separating gel. Proteins were visualized by Coomassie brilliant blue R250 staining.

Receptor binding assays. Partially purified GST-ERdef fusion proteins were diluted in TEGD buffer (10 mM Tris pH 7.6, 1.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) containing 1 mg/ml bovine serum albumin (BSA) as a carrier protein, and incubated at 4°C for 2 h with 0.1–3.5 nM [^{3}H]E2 in 1 ml glass tubes arranged in a 96-well format (Marsh Scientific, Rochester, NY). Fusion protein preparations were diluted to ensure 10,000 dpms of total binding (dilutions varied from 750–3000-fold). Binding assays were initiated by adding 240 μl of protein preparation to glass tubes containing 5 μl of DMSO and 5 μl of [^{3}H]E2; thus, the solvent concentration did not exceed 4%, unless stated otherwise. The amount of nonspecific binding was determined in the presence of a 400-fold excess of unlabeled E2. Bound [^{3}H]E2 was separated from free using a 96-well filter plate and vacuum pump harvester (Packard Instruments). Filter plates containing the protein were washed with 3 × 50 ml of TEG (10 mM Tris buffer (pH 7.6), 1.5 mM EDTA, and 10% (v/v) glycerol) and allowed to dry under continued suction for 30 s. After drying, the underside of the filter plates were sealed and 50 μl of MicroScint 20 scintillation cocktail was added to each well. Bound [^{3}H]E2 was measured using a TopCount luminescence and scintillation counter (Packard Instruments).

Competitive-ligand-binding assays were performed essentially as described above with the following modifications. Partially purified GST-ERdef fusion protein was diluted in TEGD containing 1 mg/ml BSA and was incubated with 2.5 nM [^{3}H]E2 (5 μl aliquot) and increasing concentrations of unlabeled competitor. PCB (1.0 nM–10 μM, 5 μl aliquots) at 4°C for 2 h. Bound [^{3}H]E2 was separated from free as described above. Nonspecific binding was determined in the presence of a 400-fold excess of unlabeled E2. Each treatment

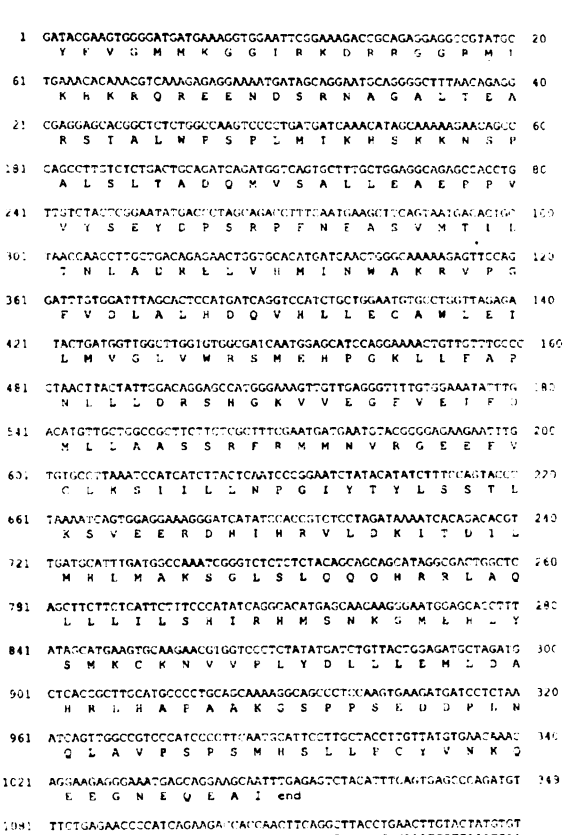

FIG. 1. Partial nucleotide sequence of the green anole ER. The first amino acid of the D domain is underlined. The boundaries of the domains were determined from an alignment to human ERα. The numbers on the left indicate the nucleotide position, and the values on the right refer to the amino acids (GenBank accession number AF095911).

was performed in quadruplicate and results are expressed as percent specific binding of [^{3}H]E2 versus log of competitor concentration. IC_{50} values were determined from nonlinear regression for single site competitive binding analysis, using Equation 1.

$$Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(X - \log IC_{50})}) \quad (1)$$

The reported IC_{50} values represent the concentration of test compound required to displace 50% [^{3}H]E2 from the GST-ER fusion proteins as compared to the 50% displacement of [^{3}H]E2 achieved by unlabeled E2. These analyses were performed using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA).

RESULTS

Isolation of Green Anole ER DEF cDNA

Figure 1 shows the partial nucleotide sequence of the green anole ER (aER). The region spanning the D, E and F domains

This is to certify that the

dissertation entitled

Molecular Basis of Estrogenic Endocrine Disruptor-Estrogen Receptor Interactions: A Comparison Among Species

presented by

Jason Bruce Matthews

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Biochemistry and Molecular Biology

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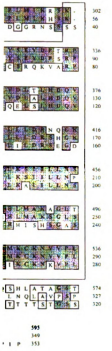

:nt to the human ERα
e D, E and F domains
terms of amino acid
as the most conserved

TABLE 2

Comparison of the Amino Acid Sequences from the D, E, and F Domains of Estrogen Receptor from Rainbow Trout Taken from Four Different Sources. Boundaries of the D, E and F Domains Were Determined from a Sequence Alignment to the Human ERα

Source of Rainbow trout ER sequence	Type of amino-acid sequence difference				
	Frame shift	Single amino acid differences			
Pakdel *et al.* (1990)	241-KPTVTWSTGQRPQ-253[a]	287-QGQTPA-292	399-RFGML-403	419-LNPGA-423	514-LNAPG-518
GenBank Accession number AF099079	18-KPYGDLEHRTAPPQ-31[b,c]	64-QGAEPPA-70	178-RFRML-182	198-LNSGA-202	293-LQSPG-297
GenBank Accession number AJ242740[d]	287-KPYGDLEHRTAPPQ-300	333-QGAEPPA-339	447-RFRML-451	467-LNSGA-471	562-LQSPG-566
GenBank Accession number AJ242741[d]	242-KPYGDLEHRTAPPQ-255	288-QGAEPPA-294	402-RFRML-406	422-LNSGA-426	517-LQSPG-521

[a] The numbers correspond to the amino acid residues in the sequence, which only includes the D, E and F domains.
[b] Amino acid residues found to be different among the sequences are shown in bold.
[c] Inserted amino acid residues are shown underlined and in bold.
[d] Recently deposited in GenBank by the author who originally published the rainbow trout ER sequence.

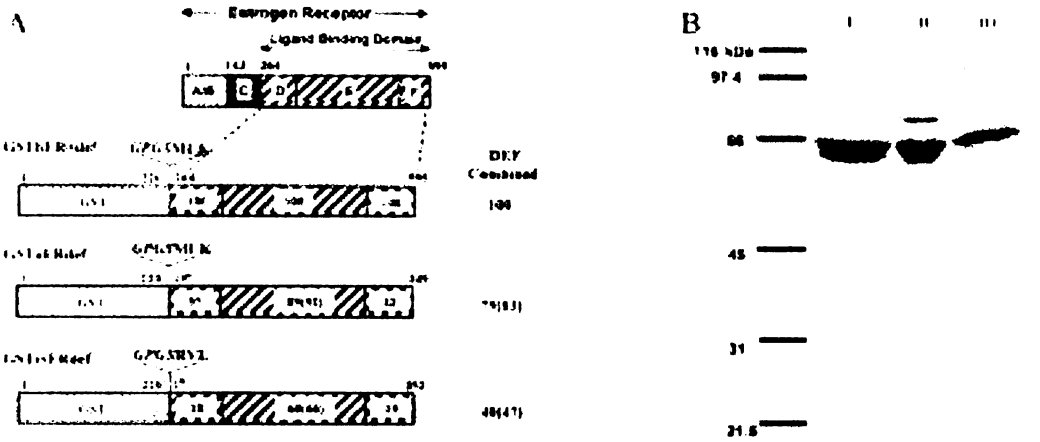

FIG. 3. (A) A schematic representation of the GST-ER fusion proteins expressed in bacteria. Amino acid residues that link the 2 proteins are provided above the transition point. The residues shown in italics represent glutathione *S*-transferase (GST). The first 3 residues of the ER ligand binding domain (DEF domains) are shown in regular text. Numbers provided above identify the amino acids used in the construction. The numbers within the domains represent % identity while those in parentheses represent % similarity to hERα. [a] Only a portion of the green anole ER sequence was cloned, and the start of the D domain corresponds to amino acid residue 19 in the cloned sequence. [b] Refers to the first amino acid of the recloned rainbow trout ER partial sequence (GenBank accession number AF099079). (B) SDS–PAGE analysis of the GST-ERdef fusion proteins purified using GSH affinity chromatography. Lane A: 5 μg of partially purified GST-hERαdef (predicted mw = 64.2 kDa. Lane B: 5 μg of partially purified GST-aERdef (predicted mw = 64.3 kDa. Lane C: 5 μg of partially purified GST-rtERdef (predicted mw = 65.5 kDa). Proteins were analyzed using a 4% stacking and a 12% separating gel stained with Coomassie brilliant blue R250.

(89% sequence identity and 92% sequence similarity) while the D (57% sequence identity) and F (32% sequence identity) domains were less conserved.

Recloning of Rainbow Trout ER DEF cDNA

The rainbow trout ER (rtER) D, E, and F domains were recloned using primers based on the previously published sequence (Pakdel *et al.*, 1990). Upon sequencing the fragment, significant differences with the published sequence were observed (Table 2), and these differences were confirmed by sequencing 7 independent clones from 3 different animals (data not shown). The recloned sequence (GenBank accession AF099079) is in agreement with 2 unpublished rtER sequences recently deposited in the GenBank database (GenBank accession AF242740 and AF242741) by the authors, who originally published the rtER sequence (Table 2). Combined, the D, E, and F domains are 40% identical and 47% similar in terms of amino acid composition, to the hERα. The E domain was the most conserved (60% sequence identity and 66% sequence similarity) while the D and F domains were less conserved (18% and 19% sequence identity, respectively).

Purification of GST-ERdef Fusion Proteins

The purity of the GST-ERdef fusion proteins was qualitatively determined to be approximately 85%, based on a Coomassie-stained SDS–PAGE analysis (Fig. 3B). All 3 fusion proteins migrated according to their predicted molecular weights (mw): GST-hERαdef (64.2 kDa), GST-aERdef protein (64.3 kDa), and GST-rtERdef (65.5 kDa). The recovery of the fusion proteins varied among species, with yields ranging from 1–3 mg/l. As shown in Figure 3B, lane I, the GST-hERαdef fusion migrated as a doublet. The higher MW band most likely represents the full length product, whereas the lower band may result from proteolytic cleavage. Although the GST-aERdef and GST-rtERdef fusions did not appear to migrate as doublets, additional higher and lower MW proteins co-purified with the fusion proteins.

Characterization of the Purified GST-ERdef Fusion Proteins

Binding affinities of the partially purified GST-ERdef fusion proteins for E2 were determined by saturation analysis (inset, Fig. 4) and linear transformation of the data (Scatchard, 1949) (Fig. 4). Differences in the amounts of receptor required to attain the desired 10,000 dpm at saturation were species-dependent and may be due, in part, to differences in protein purity, functionality, and level of expression between preparations. All GST-ERdef fusion proteins exhibited high binding affinity for E2, with dissociation constants (K_d) of 0.4 ± 0.1 nM, 0.7 ± 0.2 nM, and 0.6 ± 0.1 nM for GST-hERαdef, GST-aERdef and GST-rtERdef, respectively. These values are means ± standard deviations from 4 independent experiments.

Ligand Binding Analysis

A set of 44 PCBs, 8 Aroclors, and 9 HO-PCBs were examined for their ability to compete with [^{3}H]E2 for binding to the

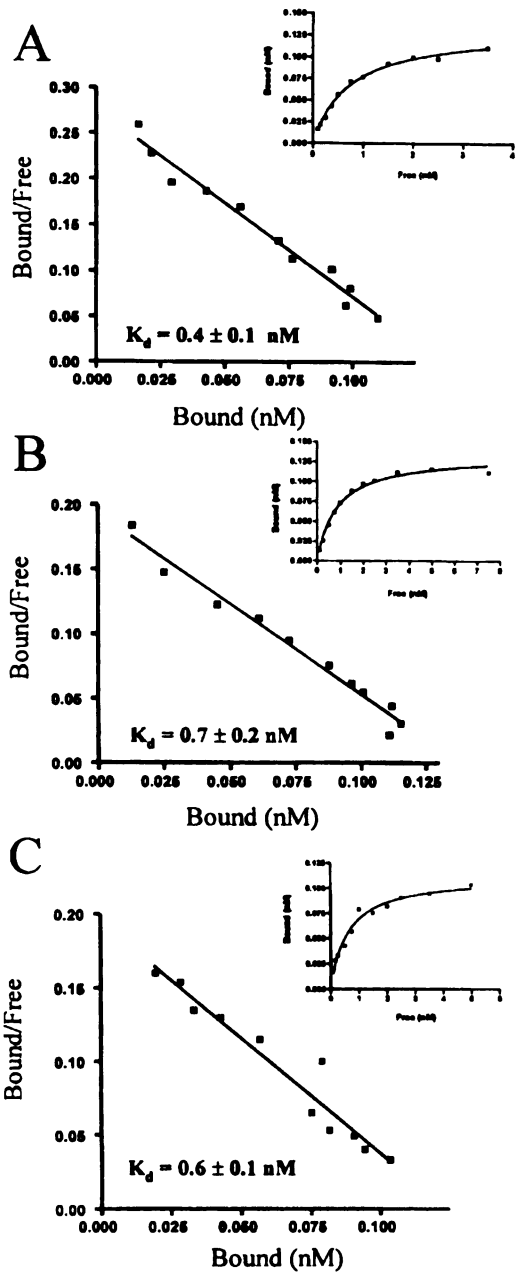

FIG. 4. Saturation analysis of (A) GST-hERαdef, (B) GST-aERdef, and (C) GST-rtERdef fusion proteins. Various concentrations of [^{3}H]E2 were incubated with known amounts of partially purified fusion protein for 2 h at 4°C as described in Materials and Methods. Saturation data shown in the inset were plotted by the method of Scatchard. The reported K_d values were averaged from 4 independent experiments.

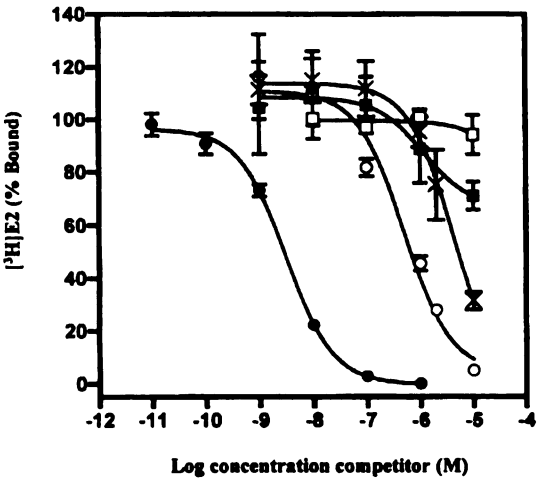

FIG. 5. Classification of PCB interactions with GST-ERdef fusion proteins. An aliquot of partially purified GST-rtERdef was incubated with 2.5 nM [^{3}H]E_2 and increasing concentrations of unlabeled competitor [filled circle] E2 (positive control)—exhibits full displacement curve with IC_{50} 2.4 ± 1.0 nM, [empty square] PCB 77—non-binder (nb), did not compete with [^{3}H]E2 for binding to GST-ERdef fusion protein, [filled square] PCB 153—weak binder (wb), only 10–30% displacement of [^{3}H]E2 from GST-ERdef fusion protein was observed at the highest dose examined, × PCB 91—congener capable of displacing at least 50% [^{3}H]E2 at the highest examined dose examined from GST-ERdef fusion proteins however characteristic displacement was not achieved IC_{50} > 10 μM, and [empty circle] PCB 184 exhibits full displacement curve with EC_{50} = 3.1 ± 0.6 nM. The results (mean and standard deviation) are from a representative experiment that was repeated 3 times.

GST-ERdef fusion proteins using a semi-high throughput competitive-binding assay. Protein preparations were incubated with 2.5 nM [^{3}H]E2 and increasing concentrations of PCBs (1.0 nM- 10 μM) for 2 h at 4°C. Kinetic studies with E2 showed that a 2-h incubation was sufficient to reach full saturation (data not shown).

Figure 5 shows binding profiles of 4 representative PCB congeners to the GST-rtERdef fusion protein, and illustrates the criteria used to classify competitive binding. The binding patterns observed for PCB 77 and PCB 153 represent PCB congeners that are classified as non-binders (nb) and weak binders (wb), respectively. A PCB congener was classified as a non-binder if less than 10% competitive binding was observed; similarly, a PCB congener was classified as a weak binder if only 10%-50% of [^{3}H]E2 was displaced at the highest concentration of competitor examined (10 μM). PCB 91 effectively displaced 50%-70% [^{3}H]E2 from the GST-rtERdef, however, a characteristic one-site competitive displacement curve was not achieved. Consequently, an IC_{50} greater than the highest concentration of test compound was ascribed. PCB 184 effectively competed with [^{3}H]E2, displacing more than 80% [^{3}H]E2 from the fusion protein and an IC_{50} value was calculated using Graphpad Prism 3.0. Concentrations greater than 10

TABLE 3

Summary of the Ability of 44 Polychlorinated Biphenyls to Compete with [^{3}H]17β-Estradiol for Binding to Recombinant Fusion Proteins Containing the Human, Green Anole and Rainbow Trout Estrogen Receptor D, E, and F Domains

PCB[a] (IUPAC)	Substitution pattern	GST-hERαdef	GST-aERdef	GST-rtERdef
Mono-*ortho*				
58	2,3,3',5'-	nb[b]	nb	wb[c]
60[d]	2,3,4,4'-	nb	nb	wb
68	2,4,3',5'-	nb	nb	wb
70[d]	2,5,3',4',-	nb	nb	wb
74[d]	2,4,5,4'-	nb	nb	wb
Di-*ortho*				
18[d]	2,5,2'-	nb	nb	wb
41	2,3,4,2'-	nb	nb	>10 μM[e]
44[d]	2,3,2',3'-	nb	nb	wb
47[d]	2,4,2',4'-	nb	nb	>10 μM
49[d]	2,4,2',5'-	nb	nb	wb
87[d]	2,3,4,2',5'-	nb	nb	nb
99[d]	2,4,4,2',4'-	nb	nb	wb
101[d]	2,4,5,2',5'-	nb	nb	wb
112	2,3,5,6,3'-	nb	nb	wb
115	2,3,4,6,4'-	nb	nb	>10 μM
128[d]	2,3,4,2',3'4'-	nb	nb	wb
138[d]	2,3,4,2',4',5'-	nb	nb	wb
153[d]	2,4,5,2',4',5'-	nb	nb	wb
158	2,3,4,6,3',4'-	nb	nb	nb
168	2,4,6,3',4',5'-	nb	nb	nb
190	2,3,4,5,6,3',4'-	nb	nb	nb
193	2,3,5,6,3'4',5'-	nb	nb	nb
194[d]	2,3,4,5,2',3',4',5'-	nb	nb	nb
Tri-*ortho*				
45[d]	2,3,6,2'-	nb	nb	>10 μM
51	2,4,2',6'-	nb	nb	>10 μM
84[d]	2,3,6,2'3'-	nb	nb	wb
91[d]	2,3,6,2',4'-	nb	nb	>10 μM
95[d]	2,3,6,2'5'-	nb	nb	wb
143	2,3,4,5,2',6'-	nb	nb	>10 μM
149[d]	2,6,2',4'5-	nb	nb	wb
151[d]	2,3,5,6,2',5'-	nb	nb	wb
173	2,3,4,5,6,2',3'-	nb	nb	>10 μM
177[d]	2,3,4,2',3',5',6'-	nb	nb	>10 μM
178	2,3,5,6,2',3',5'-	nb	nb	wb
183[d]	2,3,4,6,2',4',5'-	nb	nb	wb
187[d]	2,3,5,6,2',4',5'-	nb	nb	wb
Tetra-*ortho*				
54	2,6,2',6'-	nb	wb	>10 μM
104	2,4,6,2',6'-	>10 μM	>10 μM	1.3 ± 0.6 μM[f]
184	2,3,4,6,2',4',6'-	>10 μM	>10 μM	0.4 ± 0.1 μM
188	2,3,5,6,2',4',6'	>10 μM	>0 μM	1.3 ± 1.2 μM
Co-planar				
77[d]	3,4,3',4'-	nb	nb	nb
78	3,4,5,3'-	nb	nb	nb
126[d]	3,4,5,3',4'-	nb	nb	nb
169	3,4,5,3',4',5'	nb	nb	wb

[a] PCBs were examined at 1 nM–10 μM concentrations.

[b] Denotes non binder (nb) since no significant displace of radiolabeled 17β-estradiol was observed at the highest dose examined (10 μM), following replicate experiments.

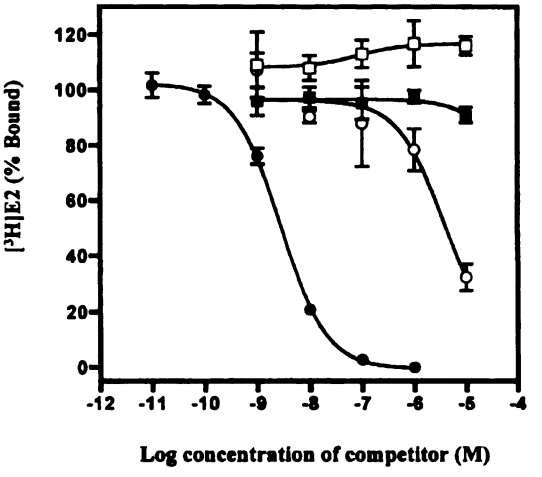

FIG. 6. Representative competitive binding curves for [filled circle] E2 to GST-hERαdef fusion protein and unlabeled PCB 47 with [empty circle] GST-rtERdef, [filled square] GST-hERαdef and [empty square] GST-aERdef fusion proteins. An aliquot of partially purified GST-ERdef fusion proteins was incubated with 2.5 nM [^{3}H]E2 and increasing concentrations of unlabeled E2 or PCB 47 as described in Materials and Methods. The results (mean and standard deviation) are from a representative experiment that was repeated 3 times.

μM were not examined, due to potential solubility limitations of the test compounds.

Table 3 summarizes the competitive binding ability of 44 PCB congeners to GST-hERαdef, GST-aERdef and GST-rtERdef fusion proteins. Only PCBs 104, 184, and 188 competed with [^{3}H]E2 for binding to GST-hERαdef and GST-aERdef fusion proteins. The remaining compounds did not significantly bind to either fusion protein.

In contrast, several PCBs competitively bound to GST-rtERdef. All 5 mono-*ortho*-substituted PCBs (PCB 58, 60, 68, 70, and 74) weakly interacted with GST-rtERdef fusion protein and displaced less than 30% of the radioligand. Nine of the 18 di-*ortho*-substituted congeners (PCB 18, 44, 49, 99, 101, 112, 128, 138, and 153) exhibited weak competitive binding pro-

[c] Denotes weak binder (wb) 10–50% displacement of radiolabeled 17β-estradiol was observed at the highest examined dose (10 μM) in two separate experiments.

[d] Environmentally relevant congeners as described in McFarland and Clarke (1989) and Hansen (1998).

[e] Uncharacteristic single site competition curve with greater than 50% displacement observed at the highest dose examined from three separate experiments, thus an IC_{50} greater than 10 μM was assigned.

[f] Characteristic single site competition curve with greater than 80% displacement of radiolabeled 17β-estradiol observed at the highest dose examined (10 μM). The IC_{50} was determined as described in Materials and Methods and represents the averages and standard deviations from 3 independent experiments.

TABLE 4

Receptor Binding Activity of 9 Hydroxylated Polychlorinated Biphenyls to Each of the Recombinant Fusion Proteins Containing the Human, Green Anole, and Rainbow Trout Estrogen Receptor D, E, and F Domains

HO-PCB	Substitution pattern	GST-hERαdef	GST-aERdef	GST-rtERdef
1[a]	2,3,5,3',4'-pentachloro-4-biphenylol	nb[b]	nb	nb
2[a]	2,3,5,2',4',5'-hexachloro-4-biphenylol	nb	nb	nb
3[a]	2,4,5,2',3',4',5'-heptachloro-3-biphenylol	nb	nb	nb
4[a]	3,5,2',3',4'-pentachloro-4-biphenylol	nb	nb	nb
5[a]	2,3,5,2',3',4'-hexachloro-4-biphenylol	nb	nb	nb
6[a]	2,3,5,2',3',4',5'-heptachloro-4-biphenylol	nb	nb	nb
7[a]	2,3,5,6,2',4',5'-heptachloro-4-biphenylol	nb	nb	nb
X[a]	2',3',4',5'-tetrachloro-4-biphenylol	0.1±0.02μM[c]	0.25±0.09μM	0.27±0.02μM
54[d]	2,6,2',6'-tetrachloro-4-biphenylol	0.5±0.02 μM	0.5 ±0.2 μM	0.3 ±0.1μM

[a] Nomenclature taken from Moore *et al.*, 1997.

[b] Denotes non binder (nb) since no significant displace of radiolabeled 17β-estradiol was observed at the highest examined dose (10 μM), following replicate experiments.

[c] Greater than 80% displacement of radiolabeled 17β-estradiol observed at the highest dose examined (10 μM). The IC_{50} was determined as described in Materials and Methods and represents the average and standard deviation from 3 independent experiments.

[d] A hydroxylated metabolite of the polychlorinated biphenyl IUPAC # 54.

files, while PCB 41, 47, and 115 displaced between 50 and 70% [^{3}H]E2 from the GST-rtERdef fusion protein. Of the 13 tri-*ortho*-substituted PCBs examined, 7 congeners (PCB 84, 95, 149, 151, 178, 183, and 187) weakly competed with [^{3}H]E2, and 6 congeners (PCB 45, 51, 91, 143, 173, and 177) displaced between 50 and 70% of [^{3}H]E2 from the GST-rtERdef fusion protein. The most active congeners contained 4 *ortho*-chloro substituents; PCB 104, 184, and 188 were strong binders, while PCB 54 only displaced 50–70% [^{3}H]E2 from the fusion protein. None of the 4 co-planar (non-*ortho* substituted) PCBs examined effectively competed with [^{3}H]E2 for binding, with the exception of PCB 169, which demonstrated a weak interaction with the GST-rtERdef fusion protein. The differential binding of PCB 47 to the GST-ER fusion proteins is shown in Figure 6. PCBs 41, 45, 51, and 91 showed similar binding curves across the species (data not shown).

Results in Table 4 summarize the competitive binding of 9 hydroxylated PCB congeners to GST-ERdef fusion proteins. HO-PCB X and HO-PCB 54 competitively bound to all 3 fusion proteins with similar affinity (IC_{50} values 0.1–0.3 μM). None of the 8 Aroclor mixtures competitively bound to any of the GST-ER fusion proteins at the highest concentration tested (10 μM). Higher concentrations were not examined due to possible solubility limitations.

Table 5 summarizes effects of DMSO on competitive binding of E2 and PCB to GST-ERdef fusion proteins. IC_{50} values for E2 were unchanged at DMSO concentrations up to 20% v/v in the assay buffer. In contrast, DMSO increased the binding affinity of select PCB congeners for the fusion proteins. Figure 7A shows the effect of DMSO on the competitive binding of E2 and PCB 184 to GST-hERαdef. At 4% DMSO, PCB 184 displayed characteristics of a weak binder; however, as the DMSO concentration increased to 20%, PCB 184 was a more effective competitor for binding to GST-hERαdef.

DISCUSSION

Recombinantly expressed human, green anole, and rainbow trout ERs were used in this study to systematically investigate the potential differences in the ligand-binding preference of structurally diverse PCBs and HO-PCBs among ERs from different species. The human ERα (hERα), considered to be the prototypical ER, was selected as the basis for all comparisons, due to the information available on its ligand-binding characteristics and the structure of the ligand-binding pocket (Brzozowski et al., 1997; Tanenbaum et al., 1998). The rainbow trout (*Onchorhynkus mykiss*) ER (rtER) was included in the study, since it represents an environmentally relevant species, and the rtER has the most divergent amino acid sequence within its ligand-binding domains (LBD, domains D, E, and F) of any cloned ER with percent identity and similarity of 40 and 47%, respectively, when compared to the hERα LBD. The ER from the green anole (*Anolis carolinensis*), a lizard commonly found throughout the southeastern United States, was also included to further investigate claims of unexpected interactions between reptilian ERs and xenobiotics (Crain et al., 1998; Crews et al., 1995; Vonier et al., 1996). The green anole ER (aER) represents the first reported complete LBD sequence for a reptile, although partial sequences have been reported (Bergeron et al., 1998; Young et al., 1995). The LBD of aER is intermediate in amino acid sequence divergence relative to hERα and rtER LBD, with percent identity and similarity of 79 and 83%, respectively, compared to hERα. These divergent sequences were selected in order to assess the appropriateness of using a single surrogate species to prioritize, identify, and assess potential EEDs. Moreover, analysis of the data and comparison of ER sequence information may identify amino acid residues that might contribute to differences in ligand preference and relative binding affinities among species.

TABLE 5

Effects of Dimethyl Sulphoxide on the Ability of Select Polychlorinated Biphenyls to Competitively Displace [^{3}H]17β-Estradiol from Recombinant Fusion Proteins Containing the Human, Green Anole, and Rainbow Trout Estrogen Receptor D, E, and F Domains

Compound	DMSO %	GST-hERαdef	GST-aERdef	GST-rtERdef
E2[a]	4	2.4 ± 1.0 nM	2.5 ± 1.3 nM	3.1 ± 0.6 nM
	10	2.8 ± 0.8 nM	2.3 ± 0.7 nM	3.1 ± 0.6 nM
	20	3.1 ± 1.1 nM	2.7 ± 0.7 nM	4.3 ± 0.4 nM
PCB 41	4	nb[b]	nb	>10 μM[c]
	10	nb	nb	6.5 ± 0.7 μM[d]
	20	wb[e]	>10 μM	4.9 ± 0.1 μM
PCB 45	4	nb	nb	>10 μM
	10	nb	nb	5.6 ± 1.4 μM
	20	wb	wb	2.5 ± 0.7 μM
PCB 47	4	nb	nb	>10 μM
	10	nb	nb	5.1 ± 0.9 μM
	20	wb	wb	6.2 ± 1.9 μM
PCB 51	4	nb	nb	>10 μM
	10	nb	nb	2.4 ± 0.6 μM
	20	wb	>10 μM	1.4 ± 0.3 μM
PCB 54	4	nb	wb	>10 μM
	10	nb	nb	1.9 ± 1.2 μM
	20	wb	wb	1.6 ± 0.3 μM
PCB 91	4	nb	nb	>10 μM
	10	nb	nb	3.8 ± 0.9 μM
	20	wb	>10 μM	2.0 ± 0.3 μM
PCB 104	4	>10 μM	>10 μM	1.3 ± 0.6 μM
	10	3.9 ± 1.1 μM	4.1 ± 1.0 μM	0.48 ± 0.02 μM
	20	1.1 ± 0.2 μM	1.1 ± 0.3 μM	0.4 ± 0.1 μM
PCB 184	4	>10 μM	>10 μM	0.4 ± 0.1 μM
	10	4.0 ± 2.2 μM	1.8 ± 0.6 μM	0.28 ± 0.02 μM
	20	0.5 ± 0.2 μM	0.7 ± 0.1 μM	0.14 ± 0.02 μM
PCB 188	4	>10 μM	>10 μM	1.3 ± 1.2 μM
	10	4.9 ± 0.9 μM	2.9 ± 1.1 μM	0.72 ± 0.01 μM
	20	1.9 ± 0.9 μM	1.4 ± 0.7 μM	0.8 ± 0.2 μM

[a] The IC_{50} was determined as described in the Materials and Methods and represents the average and standard deviation from at least 10 independent experiments.

[b] Denotes non binder (nb) since no significant displace of radioloabeled 17β-estradiol was observed at the highest examined dose (10 μM), following replicate experiments.

[c] Uncharacteristic single site competition curve with greater than 50% displacement observed at the highest dose examined from three separate experiments, thus an IC_{50} greater than 10 μM was assigned.

[d] Characteristic single site competition curve with greater than 80% displacement of radioloabeled 17β-estradiol observed at the highest dose examined (10 μM). The IC_{50} was determined as described in the Materials and Methods and represents the average and standard deviation from three independent experiments.

[e] Denotes weak binder (wb) since 10–50% displacement of radioloabeled 17β-estradiol was observed at the highest examined dose (10 μM) in two separate experiments.

The D, E, and F domains of the rtER were recloned and contained several differences when compared to the originally published sequence (Table 2). A single cytosine inserted at nucleotide position 726 of the originally published sequence (Pakdel *et al.*, 1990) resulted in a shift in the reading frame. This change was later corrected by a second insertion of a pair of cytosines at nucleotide positions 756 and 757 (Pakdel *et al.*, 1990). The insertion caused a change of 9 amino acids and resulted in the addition of a single amino acid residue within the insertion sites (Table 2). Moreover, single amino acid changes were found at various sites within the D, E, and F sequences (Table 2). It is doubtful that our sequence represents a polymorphism since the same changes were reported in two recently submitted rtER sequences in the GenBank database (GenBank accession AF242740 and AF242741).

Potential species-specific sensitivities to PCBs were investigated using the ER D, E, and F domains from mammalian (human), reptilian (green anole),and fish (rainbow trout) species expressed in bacteria as GST fusion proteins, using a semi-high throughput competitive binding assay. *In vitro* ER competitive binding assays have been well established and extensively used to investigate ER-ligand interactions. All competitive binding assays involve the displacement of a receptor-bound probe molecule by a test compound. The probe is usually [^{3}H]E2; however, fluorescently labeled high-affinity ER ligands have also been used (Bolger *et al.*, 1998). Separa-

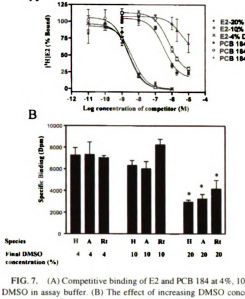

FIG. 7. (A) Competitive binding of E2 and PCB 184 at 4%, 10%, and 20% DMSO in assay buffer. (B) The effect of increasing DMSO concentration on the binding of [^{3}H]E2 to the GST-ER DEF fusion protein. A 240 μL aliquot of the GST-hERαdef (α), GST-αERdef (A) or GST-rtERdef (Rt) fusion proteins were dissolved in TEG buffer, pH 7.6 containing 1 mg/ml BSA as a carrier protein, and incubated with 2.5 nM [^{3}H]E2 and the indicated final concentration of DMSO and unlabeled competitor as indicated, for 2 h at 4°C. The amount of bound radionucleotide was compared within each species and among different concentrations of DMSO. The results (mean and standard deviation) are from a representative experiment that was repeated two times. * Indicates DMSO treatment significantly different compared to other concentrations of DMSO for each fusion protein ($p < 0.05$) using a paired Student's *t*-test.

tion of receptor-bound from free ligand can be done using dextran-coated charcoal (DCC) (Stoessel and Leclercq, 1986), hydroxyapatite (HAP) (Laws *et al.*, 1996) or by protein binding to a glass fiber membrane (Coleman *et al.*, 1997). Shortcomings of the assay include the inability to distinguish between receptor agonists and antagonists and the possibility that high concentrations of competing ligand may lead to an increase in non-specific binding (Zacharewski, 1997). However, the assay is amenable to a high throughput format and can be used to investigate direct ligand:ER interactions, which are the initial steps in many estrogenic responses.

Heterologous expression systems have been used to purify and characterize several proteins, including steroid hormone receptors (Metzger *et al.*, 1988; Wooge, 1992). Many fusion proteins exhibit activity comparable to that of their native forms (Jaglaguier *et al.*, 1996; Wittliff *et al.*, 1990). Expressing proteins as fusions facilitates the production of significant quantities of the desired regions or mutations of interest and their purification. In addition, it allows for precise control of assay conditions (protein concentration, metabolism, and background proteins) making direct comparisons among different species possible. The affinity of the bacterially expressed GST-ERdef fusion proteins for E2 was in agreement with the K_d values reported for full length ERs from human and other species (Nimrod and Benson, 1997; Pakdel *et al.*, 1990; Vonier *et al.*, 1997; Wooge, 1992). However, the affinity of the GST-rtERdef for E2 was approximately 10-fold higher than that reported for full length rainbow trout ER (Le Drean *et al.*, 1995; Pakdel *et al.*, 1990). This discrepancy may be attributed to differences in protein purity, assay conditions and the lack of accessory proteins or differences in post-translational modifications. In addition, a wide range of K_d values have been reported for some species, for example the K_d determined from Xenopus liver cytosol ER has been reported to vary from 0.5 to 15 nM (Lutz and Kloas, 1999; Westley, 1978). This suggests that differences in protein preparation and assay conditions may also contribute to the variability in the reported K_d values.

It has been demonstrated that the degree of chlorination and the substitution pattern of PCB congeners can significantly influence their estrogenic properties (Korach *et al.*, 1988; Moore *et al.*, 1997). X-ray crystallography studies have demonstrated that *ortho*-substitution causes severe conformational restriction about the inter-ring bond, and conformationally restricted hydroxylated PCBs have been shown to be effective ligands for the ER (Korach *et al.*, 1988). Quantitative structure activity relationships (QSARs) have also suggested that PCBs containing *ortho*- and *para*-chlorinated substituents are capable of binding to the ER (McKinney and Waller, 1994; Waller *et al.*, 1995). In this study, the only PCBs found to interact with GST-hERαdef and GST-αERdef receptors were three tetra-*ortho*-substituted PCBs, a penta-chlorinated PCB (PCB 104), and two hepta-chlorinated PCB (PCB 184, and PCB 188) congeners. This is in agreement with reports showing that PCB 104 was able to compete with [^{3}H]E2 for binding to mouse uterine ER and induce ER-mediated gene expression (Fielden *et al.*, 1997) and that PCB 188 and 104 induce MCF-7 cell proliferation (Andersson *et al.*, 1999). PCB 54, the fourth tetra-*ortho* substituted PCB examined, exhibited a weak interaction with the GST-αERdef protein and did not bind to the GST-hERαdef, which agrees with results reported by Arcaro *et al.* (1999) using a recombinant hERα preparation. Conversely, several PCB congeners, including PCB 104, 184, and 188, bound to the GST-rtERdef fusion protein, with the degree of interaction increasing as the number of chlorinated substituents increased. Many of the congeners that displaced at least 50% [^{3}H]E2 from the GST-rtERdef fusion protein also contained at least one *para*-chlorinated substituent in addition to the *ortho* substitutions. Of the environmentally relevant PCBs, only PCB 45, 47, 91, and 177 competitively displaced at least 50% [^{3}H]E2 from the GST-rtERdef fusion protein, however none of these congeners bound to the GST-hERαdef or GST-αERdef fusion proteins.

The differences in PCB interaction between the GST-hERαdef, GST-αERdef and GST-rtERdef fusion proteins may

be due to amino acid-sequence differences among the receptors, particularly, the amino acids that form the binding pocket. Indeed, the promiscuity of the ER has been partially attributed to the size of the ligand-binding pocket, which is approximately 2 times the volume of E2 (Brzozowski *et al.*, 1997). Amino acid sequence alignments of the ERs from different species reveal that the region of the receptor involved in ligand binding is variable. For example, the LBD of the hERα shares 90% amino acid sequence identity with the mouse ERα, 82% with the chicken ER, 79% with the green anole ER, 70% with the xenopus ER, and only 40% with the rainbow trout ER. However, identification of critical amino acid residues or motifs within the LBDs that contribute to observed differences in ligand preference and relative binding affinity through simple amino acid sequence alignment may be difficult. Despite differences in sequence identity of these ERs, the sequences from all species harbor the same 3 equivalent amino acid residues, Glu 353, Arg 394, and His 524, which participate in direct hydrogen bonds with E2 to stabilize the agonist in the binding pocket (Brzozowski *et al.*, 1997).

Rodents treated with Aroclors experience a variety of estrogenic responses, including increases in uterine glycogen content and uterine wet weight (Ecobichon and MacKenzie, 1974). However, there have been few studies examining the ER binding affinities of these mixtures. Aroclor 1221 and 1254 have been shown to weakly bind the rat uterine ER (Nelson, 1974) while Aroclors 1221, 1248, and 1268 are capable of displacing [^{3}H]E2 from the rainbow trout ER expressed in yeast (Petit *et al.*, 1997). Aroclors 1221 and 1248 (10 and 100 μM, respectively) have also been reported to induce vitellogenin synthesis in rainbow trout hepatocytes (Petit *et al.*, 1997). However, none of the Aroclors examined in this study were found to bind to any of the GST-ERdef fusion proteins. Complete congener analysis of 8 Aroclor mixtures (1221, 1232, 1242, 1016, 1248, 1254, 1260, and 1268), using capillary gas chromatography, demonstrated that PCBs 104, 184, and 188 are not detectable or are present at concentrations less than 0.05% wt (Schulz *et al.*, 1989). In addition, none of the PCB congeners found to preferentially bind to GST-rtERdef were observed to exceed 2.6% wt (Schulz *et al.*, 1989), resulting in a concentration that is unable to bind to the ER. The discrepancies between our results and those reported in the literature may be due to different assay conditions, measured endpoints, and differences in metabolic activity within the assays. It is well known that hydroxylation of select PCB congeners significantly increases their affinity for the ER (Fielden *et al.*, 1997; Korach *et al.*, 1988), thus suggesting that hydroxylation of PCB congeners plays an important role in the *in vivo* estrogenicity of Aroclor mixtures.

Although, the major HO-PCBs identified in human serum (HO-PCB 1–7) examined in this assay have been shown to significantly inhibit ER-mediated gene expression in transiently transfected MCF-7 cells (Moore *et al.*, 1997), none of the congeners were found to compete with [^{3}H]E2 for binding to the GST-ERdef fusion proteins. These results are similar to those reported by Kuiper *et al.* (1998) using baculovirus expressed hERα and rat ERβ preparations. HO-PCB 7 is a *para*-hydroxylated metabolite of PCB 187; however, the hydroxylation of this congener did not increase its affinity for any of the GST-ERdef fusion proteins. This was in contrast to the *para*-hydroxylation of PCB 54 and 104, which significantly increased the affinity of the HO-PCBs for the ER of all 3 species. HO-PCB X, which binds both mouse and rat uterine ER, also bound to all 3 GST-ERdef fusion proteins. Unlike the fully *ortho*-chloro-substituted HO-PCB congener HO-PCB 54 (2,6,2′,6′-tetrachloro-4-biphenylol), HO-PCB X (2′,3′,4′,5′-tetrachloro-4-biphenylol) contains a single *ortho* substitution, but was found to bind to all 3 GST-ERdef fusion proteins with a slightly lower affinity than HO-PCB 54. In addition, nonphenolic chloro-substituted HO-PCBs have been shown to effectively compete for binding to the ER (Kuiper *et al.*, 1998), although HO-PCB 54 consists of both phenolic and nonphenolic chloro-substitutions and competes for binding to all 3 fusion proteins. This suggests that in addition to the degree of *ortho* substitution, the chlorination pattern and position of the hydroxyl are important determinants of ER binding as previously described (Connor *et al.*, 1997; Korach *et al.*, 1988).

Increasing DMSO concentrations to 10% was found to effect ligand preference and relative binding affinity of PCBs. In contrast, it had little effect on E2 interactions with GST-ERdef fusion proteins, suggesting that DMSO may increase the solubility of PCB congeners and their availability for receptor interaction. For example, PCBs that bound weakly to the GST-ERdef fusion proteins with 4% DMSO in the assay mixture exhibited a significant increase in binding affinity in solutions containing up to 20% DMSO (Fig. 7A). However, at a final concentration of 20% DMSO, a significant decrease in total binding was observed, indicating direct effects on protein function. This observation has important implications for assessment of relative ligand-binding affinities for the ER, since organic solvent concentration may markedly influence the binding of some substances.

These results demonstrate that ERs from different species exhibit differential ligand preferences and relative binding affinities for PCBs, which can be dramatically affected by solvent concentration. Although many of the environmentally relevant PCBs did not effectively compete with [^{3}H]E2 for binding to the GST-ERdef fusion proteins, the data generated from this study can be used for further development of ER QSARs (Waller *et al.*, 1995) and also help in the derivation of species-specific QSARs (Tong *et al.*, 1997).

In summary, we report the cloning of the first complete reptilian ER DEF sequence, which has been used in a study comparing the differential binding of PCBs and HO-PCBs to the ERs from human, green anole, and rainbow trout using a semi-high throughput, competitive binding assay. Surprisingly, several examples of differences in the absolute and relative binding affinity of a number of structurally-related PCBs

among the GST-hERαdef, GST-aERdef, and GST-rtERdef proteins were observed. The lack of differences between binding affinities for the human and green anole proteins is most likely due to the higher degree of amino acid sequence identity throughout their ligand binding domains. The most notable differences were observed between the GST-rtERdef and either of the other two GST-ER fusion proteins. This may have implications for risk assessment when extrapolating data between two such divergent species as humans and rainbow trout. Studies are currently underway that examine more structurally diverse substances, including pharmaceuticals, natural products, environmental pollutants, and industrial chemicals, for potential differences in ER-binding affinity across species.

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REFERENCES

Andersson, P. L., Blom, A., Johannisson, A., Pesonen, M., Tysklind, M., Berg, A. H., Olsson, P., and Norrgren, L. (1999). Assessment of PCBs and hydroxylated PCBs as potential xenoestrogens: *In vitro* studies based on MCF-7 cell proliferation and induction of vitellogenin in primary culture of rainbow trout hepatocytes. *Arch. Environ. Contam. Toxicol.* **37,** 145–150.

Arcaro, K. F., Yi, L., Seegal, R. F., Vakharia, D. D., Yang, Y., Spink, D. C., Brosch, K., and Gierthy, J. F. (1999) 2,2′,6,6′-Tetrachlorobiphenyl is estrogenic *in vitro* and *in vivo*. *J. Cell. Biochem.* **72,** 94–102.

Bellschmiter, K., Buchert, S. H., Zell, M., Figge, K., Polzhofer, K., and Hoerschelman, H. (1981). Studies of the global baseline production, V: Monitoring the baseline pollution of the subantarctic by penguins as bioindicators. *Anal. Chem.* **309,** 1–7.

Bergeron, J. M., Gahr, M., Horan, K., Wibbels, T., and Crews, D. (1998) Cloning and *in situ* hybridization analysis of estrogen receptor in the developing gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. *Dev. Growth Differ.* **40,** 243–254.

Bergman, A., Klasson-Wehler, E. and Kuroki, H. (1994). Selective retention of hydroxylated PCB metabolites in blood. *Environ. Health Perspect.* **102,** 464–469.

Bitman, J., and Cecil, H. C. (1970). Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J. Agric. Food Chem.* **18,** 1108–1112.

Bolger, R., Wiese, T. E., Ervin, K., Nestich, S., and Checovich, W. (1998). Rapid screening of environmental chemicals for estrogen receptor binding capacity. *Environ. Health Perspect.* **106,** 551–557.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389,** 753–758.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Colborn, T., vom Saal, F. S., and Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **101,** 378–384.

Coleman, K. G., Wautlet, B. S., Morrissey, D., Mulheron, J., Sedman, S. A., Brinkley, P., Price, S., and Webster, K. R. (1997). Identification of CDK4 sequences involved in cyclin D1 and p16 binding. *J. Biol. Chem.* **272,** 18869–18874.

Connor, K., Ramamoorthy, K., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A., and Balaguer, P. (1997). Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: Structure-activity relationships. *Toxicol. Appl. Pharmacol.* **145,** 111–123.

Crain, D. A., Noriega, N., Vonier, P. M., Arnold, S. F., McLachlan, J. A., and Guillette, L. J., Jr. (1998). Cellular bioavailability of natural hormones and environmental contaminants as a function of serum and cytosolic binding factors. *Toxicol. Ind. Health* **14,** 261–273.

Crews, D., Bergeron, J. M., and McLachlan, J. A. (1995). The role of estrogen in turtle sex-determination and the effect of PCBs. *Environ. Health Perspect.* **103,** 73–77.

Ecobichon, D. J., and MacKenzie, D. O. (1974). The uterotropic activity of commercial and isomerically-pure chlorobiphenyls in the rat. Res. Commun. Chem. Pathol. Pharmacol. **9,** 85–95.

EDSTAC, F.R. (1998). Endocrine Disruptor Screening and Testing Advisory Committee Final Report: Vol. I.

Evans, R. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240,** 889–895.

Fielden, M. R., Chen, I., Chittim, B., Safe, S. H., and Zacharewski, T. R. (1997). Examination of the estrogenicity of 2,4,6,2′,6′-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2′,6′-pentachloro-4-biphenylol (HO-PCB 104), and a further chlorinated derivative, 2,4,6,2′,4′,6′-hexachlorobiphenyl. *Environ. Health Perspect.* **105,** 1238–1248.

Fitzpatrick, D. W., Picken, C. A., Murphy, L. C., and Buhr, M. M. (1989). Measurement of the relative binding affinity of zearalenone, a-zearalenol, and b-zearalenol for uterine and oviduct estrogen receptors in swine, rats, and chickens: An indicator of estrogenic potencies. *Comp. Biochem. Physiol.* **94C,** 691–694.

Gillesby, B., and Zacharewski, T. R. (1999). pS2 (TFF2) mRNA levels in human breast tumor samples: Correlation with clinical and histological prognostic markers. *Breast Cancer Res. Treat.* **1466,** 1–13.

Hansen, L. G. (1998). Stepping backward to improve assessment of PCB congener toxicities. *Environ. Health Perspect.* **106**(Suppl 1), 171–189.

Jaglaguier, S., Mesnier, D., Lager, J. L., and Auzou, G. (1996). Putative steroid binding domain of the human mineralocorticoid receptor, expressed in E. coli in the presence of heat shock proteins shows typical native receptor characteristics. *J. Steroid Biochem. Mol. Biol.* **57,** 43–50.

Katzenellenbogen, J. A. (1995) The structural pervasiveness of estrogenic activity. *Environ Health Perspect,* **103**(Suppl 7), 99–101.

Korach, K. S., Sarver, P., Chae, K., McLachlan, J. A., and McKinney, J. D. (1988). Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: Conformationally restricted structural probes. *Mol. Pharmacol.* **33,** 120–126.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139,** 4252–4263.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Laws, S. C., Carey, S. A., Kelce, W. R., Cooper, R. L., and Gray, L. E. (1996). Vinclozolin does not alter progesterone receptor (PR) function *in vivo* despite inhibition of PR binding by its metabolites in vitro. *Toxicology* **110,** 1–11.

Le Drean, Y., Kern, L., Pakdel, F., and Valotaire, Y. (1995). Rainbow trout estrogen receptor presents an equal specificity but a differential sensitivity for estrogens than human estrogen receptor. *Mol. Cell. Endocrinol.* **109,** 27–35.

Li, M. H., Zhao, Y. D., and Hansen, L. G. (1994). Multiple dose toxicokinetic influence on the estrogenicity of 2,2',4,4',5,5'-hexachlorobiphenyl. *Bull. Environ. Contam. Toxicol.* **53,** 583–590.

Lutz, I., and Kloas, W. (1999). Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. *Sci. Total Environ.* **225,** 49–57.

McFarland, V. A., and Clarke, J.,U. (1989). Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: Considerations for a congener-specific analysis. *Environ. Health Perspect.* **81,** 225–239.

McKinney, J. D., and Waller, C. L. (1994). Polychlorinated biphenyls as hormonally active structural analogues. *Environ. Health Perspect.* **102,** 290–297.

McLachlan, J. A. (1993). Functional toxicology: A new approach to detect biologically active xenobiotics. *Environ. Health Perspect.* **101,** 386–387.

McLachlan, J. A., and Korach, K. S. (1995). Symposium on estrogens in the environment: III. *Environ. Health Perspect.* **103**(Suppl. 7), 3–4.

Metzger, D., White, J. H., and Chambon, P. (1988). The human oestrogen receptor functions in yeast. *Nature* **334,** 31–36.

Moore, M., Mustain, M., Daniel, K., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A., and Balaguer, P. (1997). Antiestrogenic activity of hydroxylated polychlorinated biphenyl congeners identified in human serum. *Toxicol. Appl. Pharmacol.* **142,** 160–168.

Mullin, M. D., Pochini, C. M., McCrindle, S., Romkes, M., Safe, S. H., and Safe, L. M. (1984). High-resolution PCB analysis: Synthesis and chromatographic properties of all 209 PCB congeners. *Environ. Sci. Technol.* **18,** 468–476.

Narasimhan, T. R., Kim, H. L., and Safe, S. H. (1991). Effects of hydroxylated polychlorinated biphenyls on mouse liver mitochondrial oxidative phosphorylation. *J. Biochem. Toxicol.* **6,** 229–236.

Nelson, J. A. (1974). Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17-beta-[^{3}H]estradiol binding to rat uterine receptor. *Biochem. Pharmacol.* **23,** 447–451.

Nimrod, A. C., and Benson, W. H. (1997). Xenobiotic interaction with and alteration of channel catfish estrogen receptor. *Toxicol. Appl. Pharmacol.* **147,** 381–390.

Pakdel, F., Le Gac, F., Le Goff, P., and Valotaire, Y. (1990). Full-length sequence and in vitro expression of rainbow trout estrogen receptor cDNA. *Mol. Cell. Endocrinol.* **71,** 195–204.

Petit, F., Le Goff, P., Cravedi, J., Valotaire, Y., and Pakdel, F. (1997). Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. *J. Mol. Endocrinol.* **19,** 321–335.

Safe, S. (1993). Toxicology, structure-function relationship, and human and environmental health impacts of polychlorinated biphenyls: Progress and problems. *Environ. Health Perspect.* **100,** 259–268.

Safe, S., Washburn, K., Zacharewski, T., and Phillips, T. (1995). Synthesis and characterization of hydroxylated polychlorinated biphenyls (PCBs) identified in human serum. *Chemosphere* **31,** 3017–3023.

Scatchard, G. (1949). The attractions of proteins for small molecules. Ann. N Y Acad. Sci. **51,** 660–672.

Schulz, D. E., Petrick, G., and Duinker, J. C. (1989). Complete characterization of polychlorinated biphenyl congeners in commercial Aroclor and Clophen mixtures by multidimensional gas chromatography-electron capture detection. *Environ. Sci. Technol.* **23,** 852–859.

Schuur, A. G., Brouwer, A., Bergman, A., Coughtrie, M. W., and Visser, T. J (1998). Inhibition of thyroid hormone sulfation by hydroxylated metabolites of polychlorinated biphenyls. *Chem. Biol. Interact.* **109,** 293–297.

Stoessel, S., and Leclercq, G. (1986). Competitive binding assay for estrogen receptor in monolayer culture: Measure of receptor activation potency. *J. Steroid Biochem.* **25,** 677–682.

Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc. Natl. Acad. Sci. U S A* **95,** 5998–6003.

Tenbaum, S., and Baniahmad, A. (1997). Nuclear receptors: Structure, function, and involvement in disease. *Int. J. Biochem. Cell. Biol.* **29,** 1325–1341.

Tong, W., Perkins, R., Strelitz, R., Collantes, E. R., Keenan, S., Welsh, W. J., Branham, W. S., and Sheehan, D. M. (1997). Quantitative structure-activity relationships (QSARs) for estrogen binding to the estrogen receptor: Predictions across species. *Environ. Health Perspect.* **105,** 1116–1124.

Van den Berg, K. J., van Raaij, J. A., Bragt, P. C., and Notten, W. R. (1991). Interactions of halogenated industrial chemicals with transthyretin and effects on thyroid hormone levels *in vivo*. *Arch. Toxicol.* **65,** 15–19.

Vonier, P. M., Crain, D. A., McLachlan, J. A., Guillette, L. J., Jr., and Arnold, S. F. (1996). Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator. *Environ. Health Perspect.* **104,** 1318–1322.

Vonier, P. M., Guillette, J., L. J., McLachlan, J. A., and Arnold, S. F. (1997). Identification and characterization of estrogen and progesterone receptors from the oviduct of the American alligator (*Alligator mississippiensis*). *Biochem. Biophys. Res. Comm.* **232,** 308–312.

Waller, C. L., Minor, D. L., and McKinney, J. D. (1995). Using three-dimensional quantitative structure-activity relationships to examine estrogen receptor binding affinities of polychlorinated hydroxybiphenyls. *Environ. Health Perspect.* **103,** 702–707.

Westley, B., and Knowland, J. (1978). An estrogen receptor from *Xenopus laevis* liver possibly connected with vitellogenin synthesis. *Cell* **15,** 367–374.

Wittliff, J. L., Wenz, L. L., Dong, J., Nawaz, Z., and Butt, T. R. (1990). Expression and characterization of an active human estrogen receptor as a ubiquitin fusion protein from Escherichia coli. *J. Biol. Chem.* **265,** 22016–22022.

Wooge, C. H., Nilsson, G. M., Heierson, A., McDonnell, D. P., and Katzenellenbogen, B. S. (1992). Structural requirements for high affinity ligand binding by estrogen receptors: a comparative analysis of truncated and full length estrogen receptors expressed in bacteria, yeast, and mammalian cells. *Mol Endocrinol.* **6,** 861–869.

Young, L. J., Godwin, J., Grammer, M., Gahr, M., and Crews, D. (1995). Reptilian sex steroid receptors: Amplification, sequence, and expression analysis. *J. Steroid Biochem. Molec. Biol.* **55,** 261–269.

Zacharewski, T. (1997). In vitro bioassays for assessing estrogenic substances. *Environ. Sci. Tech.* **31,** 613–623.

CHAPTER 4

Differential estrogen receptor binding of estrogenic substances: a species comparison

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Differential estrogen receptor binding of estrogenic substances: a species comparison

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Abstract

The study investigated the ability of 34 natural and synthetic chemicals to compete with [^{3}H]17β-estradiol (E2) for binding to bacterially expressed glutathione-*S*-transferase (GST)-estrogen receptors (ER) fusion proteins from five different species. Fusion proteins consisted of the ER D, E and F domains of human alpha (GST-hERαdef), mouse alpha (GST-mERαdef), chicken (GST-cERdef), green anole (GST-aERdef) and rainbow trout ERs (GST-rtERdef). All five fusion proteins displayed high affinity for E2 with dissociation constants (K_d) ranging from 0.3 to 0.9 nM. Although, the fusion proteins exhibited similar binding preferences and binding affinities for many of the chemicals, several differences were observed. For example, α-zearalenol bound with greater affinity to GST-rtERdef than E2, which was in contrast to other GST-ERdef fusion proteins examined. Coumestrol, genistein and naringenin bound with higher affinity to the GST-aERdef, than to the other GST-ERdef fusion proteins. Many of the industrial chemicals examined preferentially bound to GST-rtERdef. Bisphenol A, 4-*t*-octylphenol and *o,p'* DDT bound with approximately a ten-fold greater affinity to GST-rtERdef than to other GST-ERdefs. Methoxychlor, *p,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE, α-endosulfan and dieldrin weakly bound to the ERs from the human, mouse, chicken and green anole. In contrast, these compounds completely displaced [^{3}H]E2 from GST-rtERdef. These results demonstrate that ERs from different species exhibit differential ligand preferences and relative binding affinities for estrogenic compounds and that these differences may be due to the variability in the amino acid sequence within their respective ER ligand binding domains.

Keywords: Estrogen receptor; Phytoestrogens; Competitive binding; Estrogenic

1. Introduction

Estrogen influences the growth, development, behavior and regulation of reproductive tissues in all vertebrates. Many of the effects of estrogens are mediated through binding to the estrogen receptor (ER). Following estrogen binding, the ER undergoes a conformational change, which facilitates chromatin binding and the modulation of estrogen responsive gene expression. The ER exists as two subtypes, ERα and ERβ, which are distinct genes that differ in their tissue distribution, and ligand preference [1]. Both receptors are modular in structure and consist of six distinct domains (A–F) [2]. The DNA-binding domain (C domain) separates the NH_2-terminal ligand-independent activation domain (A/B domains) and the COOH-terminal region, which includes a hinge region (D domain), the ligand binding domain (E domain) and a variable F domain.

It has been suggested that exposure to natural and synthetic estrogenic chemicals may adversely affect wildlife and human health [3]. There have been controversial reports of decreases in sperm production and seminal volume in humans during the past half-century [4] and increases in reproductive abnormalities in mammals [5], reptiles [6], birds [7] and several fish species [8] following exposure to environmental contaminants. However, it has also been argued that weak estrogenic chemicals do not possess sufficient potency to elicit

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these adverse health effects when compared to the intake and potency of natural estrogenic chemicals, such as phytoestrogens [9].

The diets of humans and other species consist of several natural, non-steroidal estrogenic compounds either produced by plants (phytoestrogens) or fungal molds (mycotoxins) [10]. There are three chemically distinct classes of phytoestrogens: flavonoids (e.g. genistein and naringenin), coumestans (e.g. coumesterol) and lignans (e.g. enterdiol and enterolactone), which have been associated with reproductive abnormalities in grazing animals. However, the presence of high levels of phytoestrogens in Asian diets, combined with comparatively lower rates of hormone-induced cancers prevalent in western populations, suggests that they may have a chemoprotective effect [11]. Of the mycotoxins, zearalenone and its metabolites α- and β-zearalenol are the most commonly studied and have been shown to cause reproductive problems in swine and cattle fed contaminated grain [12].

Results from several studies suggest that estrogenic compounds may exhibit differential binding preferences and relative binding affinities for both ER subtypes [13] and for ERs from different species [14]. 17β-estradiol (E2) exhibits a ten-fold lower affinity for the rainbow trout (*Onchorhynkus mykiss*) ER (rtER) than for the human ERα (hERα) [15]. Moreover, the pig ER exhibits a significantly greater affinity for α-zearalenol than does the ER from the Leghorn chicken [16]. Although, these differences may be due to the variability in the amino acid sequence within the ER ligand binding domain among species [14,17], many of these studies used different assay conditions and examined a limited set of test chemicals, making overall comparisons difficult.

Under controlled conditions potential differences in ER binding among species were further investigated using bacterially expressed glutathione-*S*-transferase (GST)-ERdef fusion proteins consisting of the D, E and F domains of human alpha, mouse alpha, chicken, green anole and rainbow trout ERs. The ability of several endogenous, synthetic and natural compounds to compete with E2 for binding to GST-ERdef fusion proteins was examined using a semi-high throughput competitive binding assay. The hERα, considered to be the prototypical ER, was selected as the basis for all comparisons due to the information available on its ligand binding characteristics and structure of its ligand binding pocket [18,19]. The mouse (*Mus musculus*) ERα and chicken (*Gallus gallus*) ER were included as representative rodent and avian ERs, respectively. The ER from the green anole (*Anolis carolinensis*; aER), a lizard commonly found throughout the southeastern United States, was also included to investigate interactions with a representative reptilian ER. In addition the aER represents the only reported complete ligand binding domain sequence for a reptile [14], although partial sequences have been previously reported [20,21]. The rtER was also examined due to its environmental relevance and because it has a highly divergent amino acid sequence within its ligand binding domain, with percent identity and similarity of 60% and 67%, respectively, when compared to the hERα.

2. Materials and methods

The steroids 17β-estradiol (1,3,5[10]-estratriene-3,17 β-diol), 17α-ethynyl estradiol (17α-ethynyl-1,3,5[10]-estratiene-3,17β-diol), estrone (1,3,5[10]-estratrien-3-ol-17-one), estriol (1,3,5[10]-estratriene-3,16α,17β-triol), β-estradiol benzoate (1,3,5[10]-estratriene-3,17β-diol 3-benzoate), DHT (dihydrotestosterone, 5α-androstan-17β-ol-3-one) and DHEA (dehydroisoandrosterone, 5-androsten-3β-ol-17-one) were purchased from Sigma (St. Louis, MO)

Synthetic estrogens tamoxifen ([Z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene), 4-hydroxytamoxifen ([Z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-buten-4-ol), diethylstilbestrol (4,4′-(1,2-diethyl-1,2-ethene-diyl)-bisphenol) were from Sigma. The antiestrogen ICI 164,384 (*n,n*-butyl-11-(3,17β-dihydroxestra - 1,3,5(10)trien - 7α - yl) - *N* - methyl - undecanamide) was a gift from Alan Wakeling of AstraZeneca (Alderley Park, UK).

The mycotoxin zearalenone (2,4-dihydroxy-6-[10-dihydroxy-6-oxo-undecyl]benzoic acid μ-lactone), its metabolites α-zearalenol (2,4-dihydroxy-6-[6α,10-dihydroxy-undecyl]benzoic acid μ-lactone), and β-zearalenol (2,4-dihydroxy-6-[6β,10-dihydroxy-undecyl]benzoic acid μ-lactone), and the flavonoids genistein (4′,5,7-trihydroxyisoflavone), naringenin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) and quercitin (3,3′,4′,5,7-pentahydroxyflavone), and β-sitosterol (22,23-dihydrostimasterol) were from Sigma. Coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Arcos Organics (Pittsburgh, PA).

The pesticide methoxychlor (1,1,1-trichloro-2,2-bis(p-methoxphenyl)ethane) and its bis-hydroxylated metabolite HPTE (2,2-bis(p-hydroxyphenyl)-1,1,1,-trichloroethane) were provided by William Kelce (Monsanto, St. Louis, MO). *o,p′*-DDT (1,1,1-trichloro-2-[2-chlorophenyl]-2-[4-chlorophenyl]ethane), *p,p′*-DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane), *o,p′*-DDE 1,1-dichloro-2-[2-chlorophenyl]-2-[4-chlorophenyl]ethylene) and *p,p′*-DDE (1,1- dichloro-bis[4-chlorophenyl] ethylene) were purchased from AccuStandard (New Haven, CT). The alkyl phenolic compound 4-*t*-octylphenol and bisphenol A (4,4′-isopropylidenediphenol) were obtained from Aldrich (Milwaukee, WI). Atrazine, simazine and chlordecone (kepone) (decachloro - octahydro - 1,3,4 - metheno - 2H - cyclobuta

(*cd*)pentalene) were from Chem-Service (West Chester, PA). Dieldrin (1,4:5,8-dimethanonaphthalene), α-endosulfan (hexachlorohexahydromethano-2,4,3-benzo-dioxathiepin-3-oxide), β-endosulfan (hexachlorohexahydromethano-2,4,3-benzo-dioxathiepin-3-oxide) were provided by S. Safe (Texas A&M University, College Station, TX). Monsanto (St. Louis, MO) and Eastman Chemical Company (Kingsport, TN) supplied butylbenzylphthalate and dibutylbenzylphthlate, respectively.

Radiolabeled [2,4,6,7,16,17-^{3}H] 17β-estradiol ([^{3}H]E2; 123 Ci/mmol) was purchased from New England Nuclear (Boston, MA). SuperScript II reverse transcriptase and Trizol Reagent were purchased from Life Technologies (Gaithesburg, MD). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA), and restriction enzymes and Taq DNA polymerase were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). All other chemicals and biochemicals were of the highest quality available from commercial sources

2.1. Recloning of chicken ER DEF domains

Total RNA from a 1-cm^3 liver section from a *G. gallus* (chicken) was isolated using Trizol Reagent according to manufacturer's instructions. One microgram of RNA was then reverse transcribed using primer PRcr-5'-aaaactcgagttatattgtattctgcatactctcctc-3' as previously described [14]. The entire cDNA products from the reverse transcription (RT) reaction were used in a PCR mixture containing 200 μM dNTPs, 2 μM primers (PRcf - 5' - aaaagaattccgaaatgatgaaacagaaacgtcaaag - 3' and PRcr) and 1.25 units of Vent DNA polymerase was amplified for 30 cycles using the following conditions: 94°C for 1 min, 62°C for 1 min and 72°C for 2 min. Sequence analysis was performed using MacVector 6.5 and the GCG Wisconsin Package (Oxford Molecular Ltd., Beaverton OR).

2.2. Construction of GST-ER DEF fusion proteins

The construction of pGEX-hERαdef, pGEX-aERdef and pGEX-rtERdef vectors has already been described [14]. The pGEX-mERαdef plasmid (mERα a.a. 268–599) was generated by PCR amplification of the plasmid pJ3MOR containing the complete mouse ER cDNA (provided by M.G. Parker; Molecular Endocrinology Research Laboratory, London, UK) using primers PRmf-5'-aaaaggatccatgttgaagcacaagcgtcagagag-3' and PRmr-5'-aaaagaattcgcgccgctcagatcgtgttgggggaagccctc-3'. The pGEX-cERdef plasmid (cER a.a. 258–589) was prepared using the products of the RT-PCR reaction described above. The mERαdef and cERdef PCR fragments were digested with the *Bam*HI/*Not*I and *Eco*RI/*Xho*I restriction enzymes and ligated into the appropriately digested GST fusion protein expression vector, pGEX6p3 (Amersham/Pharmacia; Piscataway, NJ). The PCR amplification was performed using Vent DNA polymerase (New England Biolabs) as described above. The sequence of each construct was confirmed with restriction enzyme digest and ABI/Prism automated sequencing (Perkin Elmer Applied Biosystems; Foster City, CA).

2.3. Expression and purification of GST-ERdef fusion proteins

Expression and purification of GST-ERdef fusion proteins was done as previously described [14]. Partially purified fusion proteins were separated by SDS-PAGE using a 4% stacking and 10% separating gel. Proteins were visualized by coomassie brilliant blue R250 staining.

2.4. Receptor binding assays

Receptor binding assays were performed as previously described [14]. Briefly, GST-ERdef fusion proteins were diluted in TEGD buffer (10 mM Tris pH 7.6, 1.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) containing 1 mg/ml bovine serum albumin (BSA), and incubated at 4°C for 2 h with 0.1–3.5 nM [^{3}H]E2 in 1 ml glass tubes arranged in a 96-well format (Marsh Scientific, Rochester, NY). Fusion protein preparations were diluted to ensure 10 000 dpms of total binding (varied from 750–2000-fold). Binding assays were initiated by adding 240 μl of protein preparation to glass tubes containing 5 μl of DMSO and 5 μl [^{3}H]E2, thus the concentration of solvent did not exceed 4%. Bound [^{3}H]E2 was separated from free using a 96-well filter plate and vacuum pump harvester (Packard Instruments). After drying, the filter plates were sealed and 50 μl of MicroScint 20 scintillation cocktail (Packard Instruments) was added to each well. Bound [^{3}H]E2 was measured using a TopCount luminescense and scintillation counter (Packard Instruments).

Competitive ligand binding assays were performed as described above except diluted GST-ERdef fusion protein preparations were incubated with a final concentration of 2.5 nM [^{3}H]E2 (5 μl aliquot) and increasing final concentrations of unlabeled competitor (0.1 nM–100 μM, 5 μl aliquots) at 4°C for 24 h. Each treatment was performed in quadruplicate and results are expressed as percent specific binding of [^{3}H]E2 versus log of competitor concentration. IC_{50} values were determined from non-linear regression for single site competitive binding analysis. The reported IC_{50} values represent the concentration of test compound required to displace 50% [^{3}H]E2 from the GST-ERdef fusion proteins as compared to the 50% displacement of [^{3}H]E2 achieved by unlabeled E2. Analyses were per-

formed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Recloning of chicken ERdef sequence

The recloned chicken (*G. gallus*) ERdef sequence was identical to the previously published sequence [22]. A comparison of the amino acid sequences of the cERdef with those of human, mouse, green anole and rainbow trout is shown in Fig. 1. Amino acids found to interact with E2 and/or line the ligand binding pocket are boxed. Though these residues are highly conserved among the species, there are differences. For example, Met421 in hERαdef is substituted for Phe175 in aERdef. In addition, hERαdef differs in two locations from rtERdef, in hERαdef Leu349 and Met528 replace Met93 and Ile272, in rtERdef.

3.2. Expression and saturation analysis of GST-ERdef fusion proteins

The amino acids used in the construction of the GST-ERdef fusion proteins for each species and their sequence identity compared to that of the human ERα are shown in Fig. 2. All five fusion proteins migrated according to their predicted molecular weights (MWs): GST-hERαdef (64.2 kDa), GST-mERαdef (64.4 kDa), GST-cERdef (65.2 kDa), GST-aERdef protein (64.3 kDa) and GST-rtERdef (65.5 kDa), although each appears to migrate as a doublet (Fig. 2(A)). The higher MW bands most likely represent the full-length product, whereas the lower bands may result from proteolytic cleavage [23]. In addition, higher and lower MW proteins co-purified with the proteins. The purity of the GST-ERdef fusion proteins varied among protein preparations, with yields ranging from 1 to 4 mg/l. This was evident with the GST-mERαdef preparation (Fig. 2(B) lane m) which contained lower amounts of the fusion protein, when compared to the other fusion protein preparations. However the GST-mERαdef preparations resulted in sufficient recombinant receptor to investigate the competitive binding of approximately 500 compounds per liter culture. This value varied among protein preparations, ranging from 100 to 600 compounds per liter culture.

Binding affinities of the partially purified GST-ERdef fusion proteins for E2 were determined by saturation analysis and linear transformation of the data [24] (Table 1). Differences in the amount of receptor re-

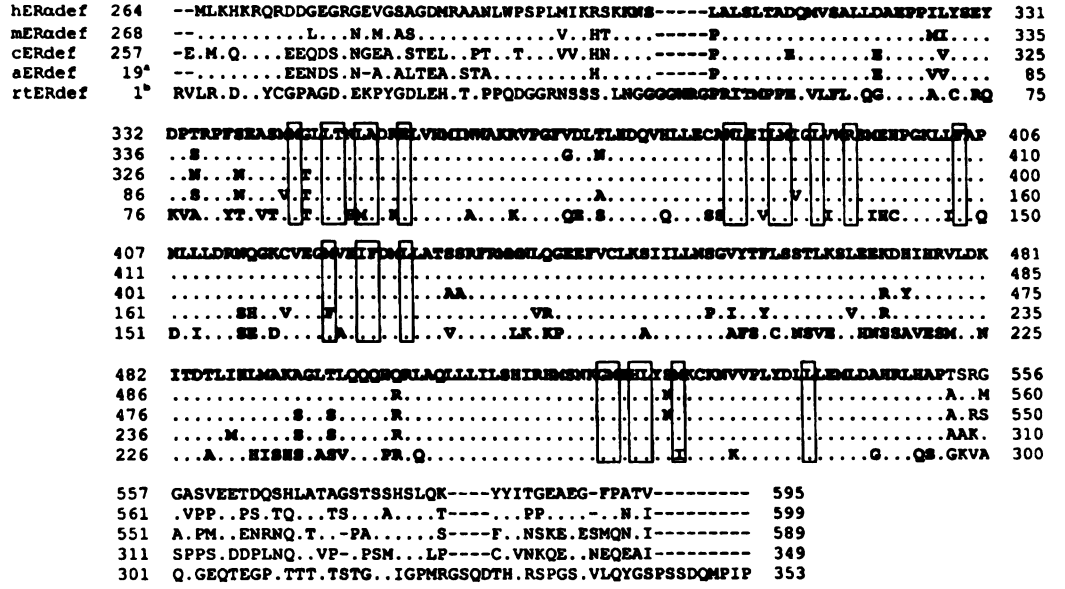

Fig. 1. Alignment of the estrogen receptor D, E and F domains from human (hERαdef), mouse (mERαdef), chicken (cERdef), green anole (aERdef) and rainbow trout (rtERdef). Numbers refer to amino acid position in the full-length sequence. Identical amino acid residues are represented as dots while missing residues are shown as dashes. The E domains are shown in bold. Residues that line the hormone binding pocket and/or interact with bound E2 are boxed. [a]Only a portion of the green anole ER sequence was cloned and the start of the D domain corresponds to amino acid residue 19 in the cloned sequence [14]. [b]Refers to the first amino acid of a recloned rainbow trout ER partial sequence [14]. This figure was modified from Pike et al. (1999) [42].

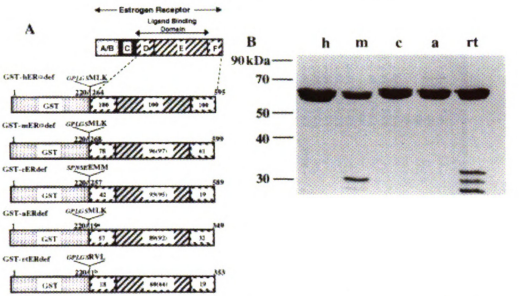

Fig. 2. (A) A schematic representation the GST-ERdef fusion proteins expressed in bacteria. Amino acid residues that link the two proteins are provided above the transition point. The residues shown in reduced font and italics represent amino acids from the linker region downstream of GST. The first three residues of the ER D domains are shown in regular text. Numbers provided above identify the amino acids used in the construction. The numbers within the domains represent the percent identity while those in parentheses represent percent similarity to hERα. *Only a portion of the green anole and rainbow trout ER sequences were cloned as previously described [14]. (B) SDS-PAGE analysis of the GST-ERdef fusion proteins purified using GSH affinity chromatography. Lane h: GST-hER9rdef (predicted molecular weight (MW) = 64.2 kDa). Lane m: GST-mER9rdef (predicted MW = 64.4 kDa). Lane c: GST-cERdef (predicted MW = 65.2 kDa). Lane a: GST-aERdef (predicted MW = 64.3 kDa). Lane rt: GST-rtERdef (predicted MW = 65.5 kDa). Each lane was loaded with 5 μg of partially purified GST-ERdef fusion protein. Proteins were analyzed using a 4% stacking and a 10% separating gel stained with coomassie brilliant blue R250.

quired to attain the desired 10 000 dpm at saturation were species-dependent and may be due, in part, to differences in protein purity, functionality and level of expression between preparations. All GST-ERdef fusion proteins exhibited high binding affinity for E2, with dissociation constants (K_d) similar to other reports (see Table 1 and references therein).

3.3. Relative binding affinities of endogenous steroids and antiestrogens

The classification of the competitive binding ability of the test compounds followed the same criteria as previously discussed [14]. Briefly, compounds were classified as non-binders (nb) if less than 10% displacement was observed or as weak binders (wb) if only 10–50% of [^{3}H]E2 was displaced at the highest concentration (100 μM) of competitor examined. For compounds that were capable of displacing greater than 50% of the [^{3}H]E2 from the GST-ERdef fusion proteins an IC_{50} value was calculated using Graphpad Prism 3.0. Concentrations greater than 100 μM were not examined due to potential solubility limitations of the test compounds.

Table 2 and Fig. 3 summarize the ability of several natural and synthetic compounds to compete with [^{3}H]E2 for binding to GST-ERdef fusion proteins. Non-binders and weak-binders were tested in a single experiment in which each treatment was performed in quadruplicate. IC_{50} values were determined for compounds that displaced at greater than 50% of the [^{3}H]E2 from the GST-ERdef fusion proteins and are shown as averages from at least two experiments. The relative binding affinities were determined for each

Table 1
Comparison of the dissociation constants (K_d) of the GST-ERdef fusion proteins with reported values

Protein	K_d (nM)	K_d reported (nM)*	References
GST-hER9rdef	0.4 ± 0.1	0.1–1.5	[27,45]
GST-mER9rdef	0.6 ± 0.2	0.1–1.4	[46,47]
GST-cERdef	0.9 ± 0.1	0.2–3.7	[16,48]
GST-aERdef	0.7 ± 0.2	0.5–17	[33,35]
GST-rtERdef	0.6 ± 0.1	0.9–6	[29,30]

* K_d values were derived from a variety of different ER sources including in vitro translated proteins, recombinant proteins expressed in bacteria, yeast and SF9 cells, and cytosol prepared from uteri, testis and liver tissue.

Table 2
IC_{50} values and relative binding affinities (RBAs) of natural and synthetic estrogenic chemicals for GST-ERdef fusion proteins following a 24-h incubation at 4°C

Test compound	GST-hERαdef		GST-mERαdef		GST-cERdef		GST-aERdef		GST-rtERdef	
	IC_{50}[a] (M)	RBA[b]	IC_{50} (M)	RBA	IC_{50} (M)	RBA	IC_{50} (M)	RBA	IC_{50}	RBA
4-Hydroxytamoxifen	$1.9 \pm 0.1 \times 10^{-9}$	155	$1.2 \pm 0.4 \times 10^{-9}$	212	$1.9 \pm 0.3 \times 10^{-9}$	168	$1.3 \pm 0.1 \times 10^{-9}$	243	$1.2 \pm 0.9 \times 10^{-9}$	272
Ethynyl estradiol	$2.3 \pm 0.1 \times 10^{-9}$	127	$2.2 \pm 0.7 \times 10^{-9}$	118	$1.9 \pm 0.1 \times 10^{-9}$	171	$2.2 \pm 0.1 \times 10^{-9}$	139	$3.1 \pm 0.9 \times 10^{-9}$	108
17β-Estradiol	$2.9 \pm 0.5 \times 10^{-10}$	100	$2.7 \pm 0.4 \times 10^{-9}$	100	$3.2 \pm 0.5 \times 10^{-9}$	100	$3.1 \pm 0.5 \times 10^{-9}$	100	$3.3 \pm 0.5 \times 10^{-9}$	100
Diethylstilbestrol	$3.2 \pm 0.1 \times 10^{-9}$	91	$3.2 \pm 0.5 \times 10^{-9}$	84	$2.5 \pm 0.6 \times 10^{-9}$	130	$2.9 \pm 1.0 \times 10^{-9}$	107	$2.0 \pm 0.1 \times 10^{-9}$	165
α-Zearalenol	$6.1 \pm 0.2 \times 10^{-9}$	48	$5.1 \pm 0.5 \times 10^{-9}$	53	$4.6 \pm 0.9 \times 10^{-9}$	70	$8.6 \pm 2.1 \times 10^{-9}$	36	$1.3 \pm 0.1 \times 10^{-9}$	267
Estrone	$6.5 \pm 0.3 \times 10^{-9}$	45	$9.5 \pm 0.8 \times 10^{-9}$	28	$6.4 \pm 0.1 \times 10^{-9}$	50	$5.1 \pm 0.1 \times 10^{-9}$	60	$2.4 \pm 0.2 \times 10^{-8}$	14
ICI 164,384	$7.0 \pm 0.3 \times 10^{-9}$	42	$5.9 \pm 0.3 \times 10^{-9}$	45	$5.2 \pm 1.0 \times 10^{-9}$	62	$1.1 \pm 0.2 \times 10^{-8}$	28	$1.0 \pm 0.7 \times 10^{-9}$	327
Estriol	$1.0 \pm 0.3 \times 10^{-8}$	28	$2.1 \pm 0.5 \times 10^{-8}$	13	$2.9 \pm 0.1 \times 10^{-8}$	11	$1.0 \pm 0.1 \times 10^{-8}$	30	$9.0 \pm 0.6 \times 10^{-8}$	3.7
β-Zearalenol	$2.3 \pm 0.3 \times 10^{-8}$	13	$2.4 \pm 1.6 \times 10^{-8}$	11	$1.4 \pm 0.1 \times 10^{-8}$	23	$7.3 \pm 1.8 \times 10^{-8}$	4.2	$3.7 \pm 0.3 \times 10^{-9}$	91
Tamoxifen	$2.8 \pm 0.4 \times 10^{-8}$	11	$2.6 \pm 0.1 \times 10^{-8}$	10	$2.1 \pm 0.1 \times 10^{-8}$	16	$3.0 \pm 0.3 \times 10^{-8}$	10	$1.3 \pm 0.1 \times 10^{-8}$	25
Estradiol benzoate	$2.8 \pm 0.5 \times 10^{-8}$	10	$2.3 \pm 0.2 \times 10^{-8}$	12	$2.2 \pm 0.1 \times 10^{-8}$	15	$2.4 \pm 0.3 \times 10^{-8}$	13	$3.7 \pm 0.5 \times 10^{-9}$	9.0
Zearalenone	$3.1 \pm 0.3 \times 10^{-8}$	9.3	$2.3 \pm 0.5 \times 10^{-8}$	12	$9.9 \pm 1.1 \times 10^{-9}$	33	$2.7 \pm 0.3 \times 10^{-8}$	12	$4.1 \pm 0.8 \times 10^{-9}$	82
HPTE	$2.5 \pm 0.8 \times 10^{-7}$	1.2	$2.2 \pm 0.2 \times 10^{-7}$	1.2	$6.8 \pm 2.2 \times 10^{-8}$	4.8	$6.4 \pm 1.7 \times 10^{-8}$	4.8	$2.4 \pm 0.1 \times 10^{-8}$	14
Coumestrol	$3.6 \pm 0.3 \times 10^{-7}$	0.81	$8.0 \pm 3.2 \times 10^{-7}$	0.33	$4.6 \pm 1.0 \times 10^{-7}$	0.70	$1.0 \pm 0.4 \times 10^{-7}$	3.1	$1.4 \pm 0.1 \times 10^{-6}$	0.24
Genistein	$6.3 \pm 0.7 \times 10^{-7}$	0.46	$8.1 \pm 0.4 \times 10^{-7}$	0.33	$4.1 \pm 0.5 \times 10^{-7}$	0.78	$2.4 \pm 0.1 \times 10^{-7}$	1.3	$7.5 \pm 0.8 \times 10^{-7}$	0.44
4-*t*-Octylphenol	$2.4 \pm 0.7 \times 10^{-6}$	0.12	$1.6 \pm 0.1 \times 10^{-6}$	0.17	$5.6 \pm 0.1 \times 10^{-7}$	0.57	$3.9 \pm 1.6 \times 10^{-6}$	0.079	$1.1 \pm 0.2 \times 10^{-7}$	3.2
Dihydrotestosterone	$5.9 \pm 0.9 \times 10^{-6}$	0.049	$6.6 \pm 1.4 \times 10^{-6}$	0.040	$3.8 \pm 0.6 \times 10^{-5}$	0.0085	$8.2 \pm 1.2 \times 10^{-7}$	0.38	$1.0 \pm 0.3 \times 10^{-5}$	0.034
Bisphenol A	$3.6 \pm 1.6 \times 10^{-5}$	0.0080	$3.1 \pm 0.7 \times 10^{-5}$	0.0086	$7.3 \pm 1.9 \times 10^{-6}$	0.044	$2.4 \pm 1.6 \times 10^{-6}$	0.13	$1.6 \pm 0.3 \times 10^{-6}$	0.21
Kepone	$4.2 \pm 1.8 \times 10^{-5}$	0.0069	$6.4 \pm 0.3 \times 10^{-5}$	0.0035	$3.0 \pm 0.1 \times 10^{-5}$	0.011	$2.7 \pm 0.7 \times 10^{-5}$	0.011	$6.2 \pm 0.4 \times 10^{-6}$	0.054
Naringenin	wb[c]	–	wb	–	$3.9 \pm 0.4 \times 10^{-5}$	0.0082	$4.7 \pm 0.8 \times 10^{-6}$	0.065	$8.7 \pm 1.3 \times 10^{-6}$	0.039
DHEA	wb	–	wb	–	wb	–	wb	–	$1.2 \pm 0.2 \times 10^{-5}$	0.028
Quercitin	wb	–	wb	–	$8.2 \pm 2.2 \times 10^{-5}$	0.0039	$1.9 \pm 0.2 \times 10^{-5}$	0.016	$8.0 \pm 2.0 \times 10^{-6}$	0.042
o,p'-DDT	wb	–	$3.6 \pm 3.5 \times 10^{-5}$	0.0073	$3.7 \pm 1.2 \times 10^{-6}$	0.086	wb	–	$7.8 \pm 0.1 \times 10^{-7}$	0.43
o,p'-DDE	wb	–	wb	–	wb	–	wb	–	$3.2 \pm 1.0 \times 10^{-6}$	0.11
p,p'-DDE	wb	–	wb	–	nb	–	wb	–	$8.0 \pm 0.6 \times 10^{-6}$	0.042
p,p'-DDT	wb	–	wb	–	wb	–	wb	–	$2.0 \pm 0.4 \times 10^{-6}$	
Butylbenzylphthalate	wb	–	wb	–	wb	–	wb	–	wb	–
Dibutylbenzylphthalate	wb	–	wb	–	wb	–	wb	–	$1.7 \pm 2.3 \times 10^{-6}$	0.20
α-Endosulfan	wb	–	wb	–	nb	–	wb	–	$2.8 \pm 1.4 \times 10^{-5}$	0.012
Methoxychlor	nb[d]	–	nb	–	wb	–	wb	–	$3.5 \pm 0.4 \times 10^{-6}$	0.95
β-Endosulfan	nb	–	nb	–	nb	–	nb	–	wb	–
Atrazine	nb	–	nb	–	nb	–	nb	–	nb	–
Simazine	nb	–	nb	–	nb	–	nb	–	nb	–
β-Sitosterol	nb	–	nb	–	nb	–	nb	–	nb	–

[a] IC_{50} values were determined from competitive binding experiments following a 24-h incubation at 4°C as described in Section 2 and represents the average and standard deviation from at least two independent experiments.

[b] Relative binding affinity (RBA) = (IC_{50} 17β-estradiol/IC_{50} compound X) × 100.

[c] Denotes weak binder (wb) since 10–50% displacement of radiolabeled 17β-estradiol was observed at the highest examined dose (100 μM).

[d] Denotes non-binder (nb) since no significant displacement of radiolabeled 17β-estradiol was observed at the highest examined dose (100 μM).

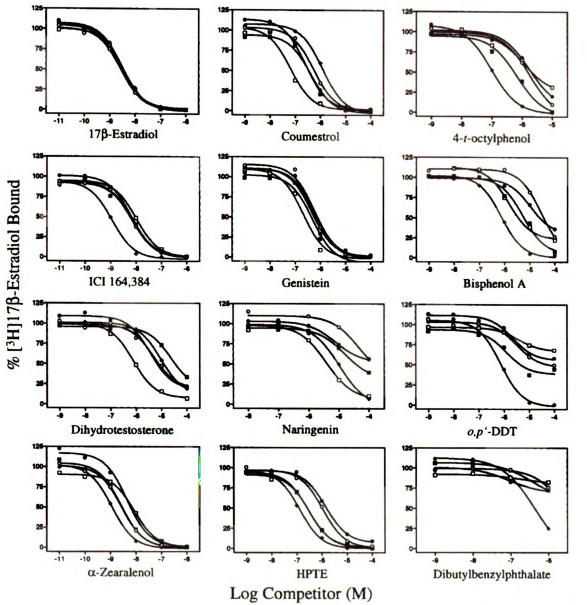

Fig. 3. Representative competitive binding curves of selected test chemicals to ● GST-hERαdef, ○ GST-mERαdef, ■ GST-cERdef, □ GST-αERdef, and ◆ GST-rtERdef fusion proteins. An aliquot of partially purified GST-ERdef fusion proteins was incubated with 2.5 nM [^{3}H]17β-estradiol and increasing concentrations of unlabeled test chemical and incubated for 24 h at 4°C as described in Section 2. The results are from a representative experiment that was repeated at least two times. Standard deviations for points on graph ranged between 5 and 15% of the mean.

compound as compared to the IC_{50} value of E2 for each GST-ERdef fusion protein. The compounds have been arranged in order of potency in comparison to GST-hERαdef. Chemicals were tested at concentrations ranging from 0.1 nM to 100 μM. This range varied depending on the competitive binding ability of the test compound.

Overall the steroidal and antiestrogenic compounds exhibited similar binding preferences and relative binding affinities for GST-ERdef fusion proteins. The E2

binding was similar across species with IC_{50} values ranging from 2.7 to 3.3 nM (Fig. 3). 4-Hydroxytamoxifen, the hydroxylated metabolite of tamoxifen, bound with greatest affinity to all 5 of the fusion proteins, with a ten- to 25-fold greater affinity than the parent compound. Diethylstilbestrol and ethynyl estradiol bound with similar affinities, 1.9–3.1 nM and 2.0–3.2 nM, respectively, to the five fusion proteins.

Though the rank order of these chemicals was similar among species, some notable differences were observed. The pure ER antagonist ICI 164,384 bound to GST-hERαdef, -mERαdef, -cERdef and -aERdef proteins with a five- to ten-fold lower affinity than to GST-rtERdef (Fig. 3). In addition, ICI 164,384 bound with higher affinity than E2 to GST-rtERdef, which was not observed with the other four fusion proteins. DHT bound to GST-aERdef with a seven- to 12-fold greater affinity than to GST-hERαdef, -mERαdef and -rtERdef, but bound with a 42-fold greater affinity than to GST-cERdef (Fig. 3), which shares 91% amino acid sequence identity to aERdef within their respective ER E, ligand binding, domains. DHEA, a precursor in the endogenous synthesis of estrogens and androgens, weakly bound to the ERs of human, mouse, chicken and green anole; however it exhibited an IC_{50} value of 12 ± 2 μM with GST-rtERdef.

3.4. Differential binding of phytoestrogens and mycotoxins

Differences in ligand preferences and binding affinities were also seen with some mycotoxins and phytoestrogens. α-Zearalenol, a hydroxylated metabolite of the mycotoxin zearalenone, consistently bound with greater affinity to the GST-rtERdef than to any other fusion proteins (Fig. 3). Similarly, the parent compound zearalenone and another metabolite, β-zearalenol, bound with greater affinity to the GST-rtERdef than to ERs from other species (Table 2). Interestingly, α-zearalenol bound with greater affinity than E2 to GST-rtERdef (2.6-fold) but bound to the other 4 fusion proteins with approximately half the affinity of E2.

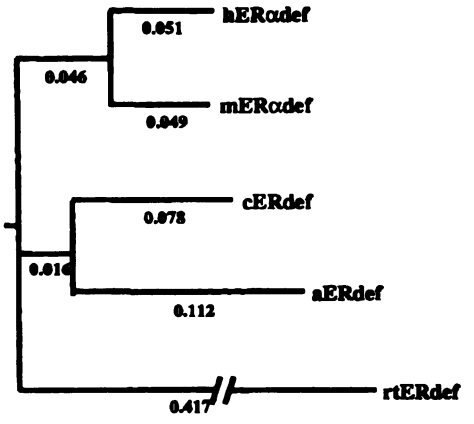

Fig. 4. Dendogram generated from the aligned ERdef sequences. The distance between nodes reflects the degree of sequence identity when doing pairwise alignments. The value of 0.1 corresponds to a difference of 10% between two sequences. This figure was generated using the ClustalW alignment function in MacVector 6.5 (Oxford Molecular Ltd.).

Overall the phytoestrogens displayed higher affinity for GST-aERdef than the other GST-ERdef fusion proteins. However, quercitin bound with slightly greater affinity to GST-rtERdef and β-sitosterol was unable to displace [^{3}H]E2 from any of the GST-ERdef fusion proteins at the highest concentration examined (100 μM; Table 2). Coumestrol bound with greatest affinity to GST-aERdef ($IC_{50} = 0.10 \pm 0.04$ μM). It exhibited similar affinity to the human, mouse, chicken and ERdef proteins (IC_{50} values ranging from 0.36 to 0.80 μM) and bound with a 14-fold lower affinity to the rainbow trout ER ($IC_{50} = 1.4 \pm 0.1$ μM; Fig. 4). Genistein exhibited similar binding affinities for all five GST-ERdef fusion proteins, but consistently bound with higher affinity to GST-aERdef (Fig. 4). Moreover, naringenin bound with highest affinity to GST-aERdef ($IC_{50} = 4.7 \pm 0.8$ μM) and slightly lower affinity to GST-rtERdef (8.7 ± 1.3 μM). However it bound with approximately a ten-fold lower affinity to GST-cERdef and bound weakly to GST-hERαdef and GST-mERαdef (Fig. 4).

3.5. Differential binding of synthetic chemicals

Overall, this class of compounds bound with greater affinity to GST-rtERdef than to any other fusion protein. Bisphenol A, 4-*t*-octylphenol and *o,p'*-DDT bound with approximately a 10-fold greater affinity to GST-rtERdef than to the ERs of the other species (Fig. 4). Complete displacement of [^{3}H]E2 by these compounds was only observed with GST-rtERdef at the highest concentration examined (100 μM). Methoxychlor, *p,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE, α-endosulfan and dieldrin were found to bind weakly to the ERs from the human, mouse, chicken and green anole. In contrast, these compounds completely displaced [^{3}H]E2 from GST-rtERdef (Table 2). Although, α-endosulfan effectively displaced [^{3}H]E2 from GST-rtERdef, its isomer β-endosulfan did not displace greater than 30% [^{3}H]E2 and was therefore classified as a weak binder. Butylbenzylphthalate and dibutylbenzylphthalate bound weakly to the ERs of the different species. However, dibutylbenzylphthalate was found to displace of 75% [^{3}H]E2 from GST-rtERdef ($IC_{50} = 1.7 \pm 2.3$ μM) at the highest concentration (1 μM) examined (Fig. 3). A visible precipitate was observed at concentrations greater than 10 μM and 1 μM, for butylben-

zylphthalate and dibutylbenzylphthalate, respectively, thus higher concentrations were not examined. Finally, two chloro-*S*-triazines (Atrazine and Simazine) were unable to displace [^{3}H]E2 from any of the GST-ERdef fusion proteins at the highest concentration examined (100 μM).

The time to equilibration for steroid receptor:competitor complexes has been reported to be greater than 16 h at lower temperatures [25]. Since the competitive ligand-binding assay used a 24-h incubation time it is unlikely that the observed differences in binding affinities were due to a lack of equilibration. However when this data set was examined following a 2-h incubation at 4°C, the binding profiles of most chemicals and differences in ligand preferences, and binding affinities were similar to that of the 24 h incubation time, though there were some exceptions (unpublished data).

4. Discussion

Previous studies using a variety of protein preparations and assay conditions suggest that there may be differences in the absolute and relative binding affinities of structurally diverse estrogenic chemicals to ERs from different species. In order to investigate species-specific ligand preferences, and differences in relative and absolute binding affinities, GST-ERdef fusion proteins consisting of mammalian (human and mouse), avian (chicken), reptilian (green anole) and fish (rainbow trout) ERs were constructed.

It has been reported that truncated forms of the glucocorticoid receptor can effect protein stability and receptor function [26]. However, truncated forms of nuclear receptors overexpressed in heterologous expression systems have been previously shown to exhibit comparable affinities and ligand preferences relative to their native forms [27,28]. Heterologous expression of GST-ERdef facilitates purification of these fusion proteins and allows for precise control of the competitive binding assay conditions (e.g. protein concentration, metabolism, non-specific binding, background/accessory proteins), making direct comparisons possible.

The affinity of the bacterially expressed GST-ERdef fusion proteins for E2 was similar to the K_d values reported for full length ERs (Table 2). However, the affinity of the GST-rtERdef for E2 was approximately ten-fold higher than that reported for full length rainbow trout ER [29], but was in agreement with reports using [^{3}H]moxestrol [30]. In general, the reported K_d values for some species vary considerably, which may be due to the use of different assays such as dextran-coated charcoal and hydroxylapatite methods. For example, the K_d value determined from *Xenopus* liver cytosol ER has been reported to vary from 0.5 to 15 nM [31,32]. Similarly, the K_d value determined from turtle ER using two different receptor sources, hepatic and testis cytosol, varies from 0.7 to 17 nM, respectively [33,34]. This suggests that differences in protein preparation, assay conditions and assay methods may contribute to the variability in the reported K_d values.

The ER has been shown to bind several structurally diverse chemicals. This property appears to be unique among nuclear receptors and is also true for mammalian ERα and ERβ subtypes [13] as well as for ERs from non-mammalian species [15,35]. Crystal structures of hERα E domain in complex with E2 [18,19] support ER-E2 interaction models generated from binding studies, structure activity relationships and three-dimensional homology models using crystallographic data from other nuclear receptors [36–38]. E2 binding is achieved by a combination of specific hydrogen bonding interactions and the hydrophobic nature of the binding pocket. The promiscuity of the ER has been partially attributed to the size of the ligand binding pocket, which is almost twice the volume of E2 [19]. Despite the differences in sequence identity among species [14,17], ERs from all species harbor the same three equivalent amino acids to hERα (Glu353, Arg394 and His524) that participate in direct hydrogen bonds and stabilize E2 in the binding pocket [18,19]. However, differential binding of several natural and synthetic chemicals to hERα and hERβ, as well as to ERs from different species have been reported [14–16,35,39–41]. This suggests that additional amino acid residues may also play a role in determining ligand preference and relative binding affinity.

Genistein has been shown to preferentially bind with 30-fold greater affinity to hERβ than hERα [13]. The recent report of the crystal structure of ERβ in complex with genistein has suggested that this ligand preference may be attributed to two conservative mutations within the binding pocket that may be responsible for further stabilizing the hERβ-genistein complex [42].

Although many of the compounds examined in this study, including E2, bound with similar affinity to all five GST-ERdef fusion proteins, some notable differences were reported. The most striking differences in relative binding affinities were seen with GST-aERdef and GST-rtERdef. Comparison of the amino acid differences within their respective ligand binding domains suggests that aERdef:Phe175, rtERdef:Met93 and rtERdef:Ile272 may contribute to the observed differences in ligand preference and relative binding affinities. Preliminary mutagenesis studies indicate that these residues influence relative and absolute binding affinities of a subset of estrogenic compounds (Matthews, J.B. et al. manuscript in preparation). These residues may change the hydrophobicity and volume of the binding pocket as well as result in unique ligand-residue

interactions. Recent crystallography data has demonstrated that each of these parameters can influence ligand binding. For example, the volume of the probe-occupied ligand pocket of ERα-E2 crystal complex has been determined to be 490 Å while that of ERβ-genistein is 390 Å with the reduction being primarily due to the replacement of the Leu384 in hERα with a bulkier Met336 residue in hERβ [42]. This allows the residues that line the pocket to pack more tightly around genistein, stabilizing the ligand in the binding pocket in ERβ [42].

There were no obvious relationships between sequence identity and binding affinity. GST-rtERdef has the lowest sequence identity compared to GST-hERdef, but this was not predictive of the binding affinity of a compound for GST-rtERdef. For example, 4-*t*-octylphenol bound with greater affinity to GST-rtERdef ($IC_{50} = 0.11 \pm 0.02$ μM) compared to GST-hERαdef ($IC_{50} = 2.4 \pm 0.7$ μM) while the rank order binding affinities were reversed for coumestrol (IC_{50} values of 0.36 ± 0.03 μM vs. 1.4 ± 0.1 μM for GST-hERαdef and GST-rtERdef, respectively). However, some patterns in the relative binding affinity data were observed. Cluster analysis based on amino acid sequence identity suggested that hERαdef and mERαdef shared greater similarity than cERdef and aERdef, with rtERdef being the most divergent when compared to the other ER sequences (Fig. 4). In general, ERdef proteins with greater similarity exhibited similar relative binding affinities as illustrated in Fig. 3, with some notable exceptions. Although cERdef and aERdef shared the greatest similarity, DHT exhibited a 42-fold difference in relative binding affinity between the two species. The difference was only seven-, eight- and 12-fold for GST-hERdef, GST-mERdef and GST-rtERdef, respectively. GST-rtERdef, which has the most divergent amino acid sequence according to the cluster analysis, exhibited the greatest promiscuity in its ligand preference, further supporting the hypothesis that structural differences within the ligand binding domains among ERs of different species influences ligand preference and relative binding affinity.

The results demonstrate that ERs from human, mouse, chicken, green anole and rainbow trout exhibit differential ligand preferences and relative binding affinities for a number of natural and synthetic compounds. This data can be used to further develop ER quantitative structure activity relationships (QSARs) [43] and to evaluate the feasibility of species-specific ER QSARs [44]. Although the majority of substances examined in this study exhibited comparable relative binding affinities across ERs, a significant number of differences were observed. The relative binding affinities of the GST-rtERdef, which has the greatest amino acid variation in its E domain relative to the other species examined, exhibited the most striking differences. The rtER also had the greatest ligand promiscuity, binding a significantly greater number of structurally diverse estrogenic compounds. However, pharmacokinetic and pharmacodynamic differences between species make it unlikely that differences in binding affinities for individual estrogenic compounds would be observed in vivo. Nevertheless, in the absence of structural data for natural and synthetic ligands, this cross species comparison provides valuable insights into potentially important residues that may play critical roles in the interaction between structurally diverse ligands and the ER binding pocket.

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References

[1] S. Mosselman, J. Polman, R. Dijkema, ER beta: identification and characterization of a novel human estrogen receptor, FEBS Lett. 392 (1996) 49–53.
[2] R. Evans, The steroid and thyroid hormone receptor superfamily, Science 240 (1988) 889–895.
[3] T. Colborn, F.S. vom Saal, A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environ. Health Perspect. 101 (1993) 378–384.
[4] E. Carlsen, A. Giwercman, N. Keiding, N.E. Skakkebaek, Declining semen quality and increasing incidence of testicular cancer: is there a common cause?, Environ. Health Perspect. 103 (1995) 137–139.
[5] P.J. Reijnders, Reproductive failure in common seals feeding on fish from polluted coastal waters, Nature 324 (1986) 456–457.
[6] L.J.J. Guillette, T.S. Gross, G.R. Masson, J.M. Matter, H.F. Percival, A.R. Woodward, Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida, Environ. Health Perspect. 102 (1994) 680–688.
[7] D.M. Fry, Reproductive effects in birds exposed to pesticides and industrial chemicals, Environ. Health Perspect. 103 (1995) 165–171.
[8] H. Von Westernhagen, H. Rosenthal, V. Dethlefsen, W. Ernst, U.L. Harms, P.D. Hansen, Bioaccumulating substances and reproductive success in the Baltic flounder, *Platichthys flesus*, Aquatic Tox. 1 (1981) 85–99.
[9] S.H. Safe, Environmental and dietary estrogens and human health: is there a problem? Environ. Health Perspect. 103 (1995) 346–351.
[10] K.S. Korach, S. Migliaccio, V.L. Davis, in: P.L. Munson (Ed.), Principles of Pharmacology — Basic Concepts and Clinical Applications, Chapman and Hall, New York, 1994, pp. 809–825.
[11] M.J. Messina, V. Persky, K.D. Setchell, S. Barnes, Soy intake and cancer risk: a review of the in vitro and in vivo data, Nutr. Cancer 21 (1994) 113–131.
[12] C.J. Mirocha, S.V. Pathre, C.M. Christensen, in: Y. Pomeranz (Ed.), Advances in Cereal Chemistry, American Association of Cereal Chemists, St. Paul, MI, 1980, pp. 159–225.

[13] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (1998) 4252–4263.

[14] J.B. Matthews, T.R. Zacharewski, Differential binding affinities PCBs, HO-PCBs and Aroclors with recombinant human, rainbow trout (*Onchorhynkiss mykiss*) and reptilian (*Anolis carolinensis*) estrogen receptors using a semi-high throughput competitive binding assay, Toxicol. Sci. 53 (2000) 326–339.

[15] Y. Le Drean, L. Kern, F. Pakdel, Y. Valotaire, Rainbow trout estrogen receptor presents an equal specificity but a differential sensitivity for estrogens than human estrogen receptor, Mol. Cell. Endocrinol. 109 (1995) 27–35.

[16] D.W. Fitzpatrik, C.A. Picken, L.C. Murphy, M.M. Buhr, Measurement of the relative binding affinity of zearalenone, α-zearalenol and β-zearalenol for uterine and oviduct estrogen receptors in swine, rats and chickens: an indicator of estrogenic potencies, Comp. Biochem. Physiol. 94C (1989) 691–694.

[17] M.R. Fielden, I. Chen, B. Chittim, S.H. Safe, T.R. Zacharewski, Examination of the estrogenicity of 2,4,6,2,6-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2,6-pentachloro-4-biphenylol (HO-PCB 104), and a further chlorinated derivative,2,4,6,2,4,6-hexachlorobiphenyl, Environ. Health Perspect. 105 (1997) 1238–1248.

[18] D.M. Tanenbaum, Y. Wang, S.P. Williams, P.B. Sigler, Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains, Proc. Natl. Acad. Sci. USA 95 (1998) 5998–6003.

[19] A.M. Brzozowski, A.C. Pike, Z. Dauter, R.E. Hubbard, T. Bonn, O. Engstrom, L. Ohman, G.L. Greene, J.A. Gustafsson, M. Carlquist, Molecular basis of agonism and antagonism in the oestrogen receptor, Nature 389 (1997) 753–758.

[20] L.J. Young, J. Godwin, M. Grammer, M. Gahr, D. Crews, Reptilian sex steroid receptors: amplification, sequence and expression analysis, J. Steroid Biochem. Mol. Biol. 55 (1995) 261–269.

[21] J.M. Bergeron, M. Gahr, K. Horan, T. Wibbels, D. Crews, Cloning and in situ hybridization analysis of estrogen receptor in the developing gonad of the red-eared slider turtle, a species with temperature-dependent sex determination, Dev. Growth Differ. 40 (1998) 243–254.

[22] A. Krust, S. Green, P. Argos, V. Kumar, P. Walter, J. Bornert, P. Chambon, The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors, EMBO J. 5 (1986) 891–897.

[23] D.A. Seielstad, K.E. Carlson, J.A. Katzenellenbogen, P.J. Kushner, G.L. Greene, Molecular characterization by mass spectrometry of the human estrogen receptor ligand-binding domain expressed in *Escherichia coli*, Mol. Endocrinol. 9 (1995) 647–658.

[24] G. Scatchard, The attractions of proteins for small molecules, Ann. New York Acad. Sci. 51 (1949) 660–672.

[25] P. Aranyi, Kinetics of the hormone-receptor interaction. Competition experiments with slowly equilibrating ligands, Biochim. Biophys. Acta 628 (1980) 220–227.

[26] M. Xu, P.K. Chakraborti, M.J. Garabedian, K.R. Yamamoto, S.S. Simons, Modular structure of glucocorticoid receptor domains is not equivalent to functional independence. Stability and activity of the steroid binding domain are controlled by sequences in separate domains, J. Biol. Chem. 271 (1996) 21430–21438.

[27] C.H. Wooge, G.M. Nilsson, A. Heierson, D.P. McDonnell, B.S. Katzenellenbogen, Structural requirements for high affinity ligand binding by estrogen receptors: a comparative analysis of truncated and full length estrogen receptors expressed in bacteria, yeast, and mammalian cells, Mol. Endocrinol. 6 (1992) 861–869.

[28] S. Jaglaguier, D. Mesnier, J.L. Lager, G. Auzou, Putative steroid binding domain of the human mineralocorticoid receptor, expressed in *E. coli* in the presence of heat shock proteins shows typical native receptor characteristics, J. Steroid Biochem. Mol. Biol. 57 (1996) 43–50.

[29] F. Pakdel, F. Le Gac, P. Le Goff, Y. Valotaire, Full-length sequence and in vitro expression of rainbow trout estrogen receptor cDNA, Mol. Cell Endocrinol. 71 (1990) 195–204.

[30] R.M. Donohoe, L.R. Curtis, Estrogenic activity of chlordecone, *o,p'*-DDT and *o,p'*-DDE in juvenile rainbow trout: indication of vitellogenesis and interaction with hepatic estrogen binding sites, Aquatic Toxicol. 36 (1996) 31–52.

[31] B. Westley, J. Knowland, An estrogen receptor from *Xenopus laevis* liver possibly connected with vitellogenin synthesis, Cell 15 (1978) 367–374.

[32] I. Lutz, W. Kloas, Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding, Sci. Total Environ. 225 (1999) 49–57.

[33] S.M. Ho, S. Fehrer, M. Yu, L.C. Liang, D. Press, High-affinity binding of [^{3}H]estradiol-17 beta by an estrogen receptor in the liver of the turtle, Gen. Comp. Endocrinol. 70 (1988) 382–394.

[34] P. Mak, S.M. Ho, I.P. Callard, Characterization of an estrogen receptor in the turtle testis, Gen. Comp. Endocrinol. 52 (1983) 182–189.

[35] P.M. Vonier, J.L.J. Guillette, J.A. McLachlan, S.F. Arnold, Identification and characterization of estrogen and progesterone receptors from the oviduct of the American alligator (*Alligator mississippiensis*), Biochem. Biophys. Res. Commun. 232 (1997) 308–312.

[36] G.M. Anstead, K.E. Carlson, J.A. Katzenellenbogen, The estradiol pharmacophore: ligand structure–estrogen receptor binding affinity relationships and a model for the receptor binding site, Steroids 62 (1997) 268–303.

[37] J.M. Wurtz, U. Egner, N. Heinrich, D. Moras, A. Mueller-Fahrnow, Three-dimensional models of estrogen receptor ligand binding domain complexes, based on related crystal structures and mutational and structure-activity relationship data, J. Med. Chem. 41 (1998) 1803–1814.

[38] D. Lewis, M. Parker, R. King, Molecular modelling of the human estrogen receptor and ligand interactions based on site-directed mutagenesis and amino acid sequence homology, Steroid Biochem. Mol. Biol. 52 (1995) 55–65.

[39] G.G. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, J.A. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta, Endocrinology 138 (1997) 863–870.

[40] T. Barkhem, B. Carlsson, Y. Nilsson, E. Enmark, J. Gustafsson, S. Nilsson, Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists, Mol. Pharmacol. 54 (1998) 105–112.

[41] K. Connor, J. Howell, I. Chen, H. Liu, K. Berhane, C. Sciarretta, S. Safe, T. Zacharewski, Failure of chloro-*S*-triazine-derived compounds to induce estrogen receptor-mediated responses in vivo and in vitro, Fund. Appl. Toxicol. 30 (1995) 93–101.

[42] A.C. Pike, A.M. Brzozowski, R.E. Hubbard, T. Bonn, A.G. Thorsell, O. Engstrom, J. Ljunggren, J. Gustafsson, M. Carlquist, Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist, EMBO J. 18 (1999) 4608–4618.

[43] C.L. Waller, D.L. Minor, J.D. McKinney, Using three-dimensional quantitative structure–activity relationships to examine estrogen receptor binding affinities of polychlorinated hydroxybiphenyls, Environ. Health Perspect. 103 (1995) 702–707.

[44] W. Tong, R. Perkins, R. Strelitz, E.R. Collantes, S. Keenan, W.J. Welsh, W.S. Branham, D.M. Sheehan, Quantitative structure-activity relationships (QSARs) for estrogen binding to the

estrogen receptor: predictions across species, Environ. Health Perspect. 105 (1997) 1116–1124.

[45] R.L. Eckert, B.S. Katzenellenbogen, Physical properties of estrogen receptor complexes in MCF-7 human breast cancer cells. Differences with anti-estrogen and estrogen, J. Biol. Chem. 257 (1982) 8840–8846.

[46] G.B. Tremblay, A. Tremblay, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, F. Labrie, V. Giguere, Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta, Mol. Endocrinol. 11 (1997) 353–365.

[47] T. Horigome, T.S. Golding, V.E. Quarmby, D.B. Lubahn, K. McCarty, Sr, K.S. Korach, Purification and characterization of mouse uterine estrogen receptor under conditions of varying hormonal status, Endocrinology 121 (1987) 2099–2111.

[48] E.R. Mulvihill, R.D. Palmiter, Relationship of nuclear estrogen receptor levels to induction of ovalbumin and conalbumin mRNA in chick oviduct, J. Biol. Chem. 252 (1977) 2060–2068.

CHAPTER 5

Ability of several natural and synthetic estrogenic chemicals to induce gene expression mediated by estrogen receptors from several vertebrate species

Abstract

The ability of 15 natural and synthetic estrogenic compounds to induce gene expression mediated through ERα and β isoforms and ERs from different vertebrate species was examined. MCF-7 cells were transiently transfected with a Gal4-ER chimeric receptor consisting of the D, E and F domains of the human α (Gal4-hERαdef), mouse α (Gal4-mERαdef), mouse β (Gal4-mERβdef), chicken (Gal4-cERdef), green anole (Gal4-aERdef), xenopus (Gal4-xERdef) or rainbow trout α ERs (Gal4-rtERαdef). The 17β-estradiol (E2) induced reporter gene expression was similar among the different constructs with EC_{50} values ranging from 0.05 to 0.7 nM. Intriguingly, the E2 induced response mediated by Gal4-rtERαdef was 2 orders of magnitude lower, with an EC_{50} value of 28 nM at 37°C. However, at 20°C only a 9-fold difference in EC_{50} values was observed. As a result, the ability of test compounds to induced Gal4-rtERαdef-mediated gene expression was examined at both 37°C and 20°C. Although, the response of E2 was similar among the ERs, many differences were observed. Coumestrol induced Gal4-mERβdef- and Gal4-aERdef-mediated reporter gene expression 164-fold and 8-fold greater compared to the other Gal4-ERs, respectively. In contrast to other Gal4-ERs, α-zearalenol induced Gal4-rtERαdef-mediated reporter gene expression at lower concentrations than E2. In general, the gene expression data correlated well with the competitive binding results presented in chapter 4, with a Pearson r value of 0.86. Overall the results show that certain estrogenic compounds exhibit a differential ability to induce reporter gene activity mediated by ERs from different vertebrate species. In

addition, these data also highlight the importance of incubation temperature when examining rtERα-mediated activity.

Introduction

Several studies have reported differences in relative binding affinity (RBA) of estrogenic endocrine disruptors (EEDs) for different ER isoforms and ERs from different species (1,2). For example, in chapter 3 we have demonstrated that the rtERα exhibits greater affinity for several PCB congeners than either the human or anole ERs. In chapter 4, several differences in RBA of EEDs for ERs from different classes of vertebrate species were observed. However, in other studies the RBAs of several EEDs did not correlate with their ability to induce the expression of estrogen responsive complement C3 mRNA in treated rat endometrial adenocarcinoma cells (3), suggesting that RBA is not a good predictor of estrogenic activity.

This may not be surprising since ligand binding to the ER is the first event in a complex mechanism leading to changes in gene expression. The transcriptional activity of the ER is mediated by two separate activation functions, an NH_2-terminal ligand-independent activation function (AF-1) and the ligand-dependent activation function (AF-2) located in the ligand binding domain. Although the mechanism by which the AF-2 region transmits ligand signals to the basal transcriptional machinery is poorly understood, several proteins that interact with the AF-2 region in a ligand-dependent manner have been identified (4,5). These proteins, collectively termed cofactors, function as coactivators or corepressors to induce or inhibit gene expression, respectively. After estrogen binding and subsequent dimerization, the ER undergoes a conformational change, which allows it to bind to its cognate DNA target site, referred to as estrogen responsive elements (EREs) located in the regulatory region of estrogen-inducible promoters resulting in the transcriptional regulation of target genes (6). In addition, the

ligand-induced conformational change exposes critical residues within the AF-2 region that are essential for cofactor recruitment. Functional and structural studies have shown that coactivators interact with the AF-2 region via short leucine motifs (ie. LxxLL or NR-box) to transduce the ligand signal to the basal transcriptional machinery. Several structurally distinct classes of coactivator proteins have been identified. For example, the steroid receptor coactivator (SRC) family of coactivators (7) is a family of 160 kDa molecular weight proteins that were the first coregulators shown to interact with nuclear receptors. Members of this family include, SRC-1 (7,8), SRC-2 (9,10), and SRC-3 (11-15) and they form a mutiprotein complex with CBP/p300 (12,14). Recent studies have shown that the ability of the ER to recruit SRC coactivators is dependent on the structural characteristics of the ligand (16).

There are reports in which RBAs of EEDs do not correlated with transactivation ability (1,17). For example, despite the apparent greater affinity of genistein for ERβ, genistein exhibits a similar ability to induce reporter gene expression mediated by either ERβ or ERα (1). In addition, we have observed that bisphenol A also binds to ERβ with greater affinity (10-fold) than ERα, but only exhibited a 2-fold greater ability to induce gene expression mediated by either isoform (chapter 7).

Since we have examined the ability of several EEDs to compete for binding to ERs from different species, it was of interest to examine whether differences in RBA correlated with the ability of EEDs to induce gene expression mediated by ERαs from five different vertebrate classes and a representative mammalian ERβ.

Materials and methods

Chemicals and biochemicals

17β-estradiol (1,3,5[10]-estratriene-3,17β-diol), DHT (dihydrotestosterone, 5α-androstan-17β-ol-3-one), diethylstilbestrol (4,4′-(1,2-diethyl-1,2-ethene-diyl)-bisphenol), α-zearalenol (2,4-dihydroxy-6-[6α,10-dihydroxy-undecyl]benzoic acid μ-lactone), β-zearalenol (2,4-dihydroxy-6-[6β,10-dihydroxy-undecyl]benzoic acid μ-lactone), genistein (4′,5,7-trihydroxyisoflavone), naringenin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) and β-sitosterol (22,23-dihydrostimasterol) were purchased from Sigma (St. Louis, MO). Coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Arcos Organics (Pittsburgh, PA). Methoxychlor (1,1,1-trichloro-2,2-bis(p-methoxphenyl)ethane) was provided by William Kelce (Monsanto, St. Louis, MO). o,p′-DDT (1,1,1-trichloro-2-[2-chlorophenyl]-2-[4-chlorophenyl]ethane), and p,p′-DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane), were purchased from AccuStandard (New Haven, CT). The alkyl phenolic compound 4-*t*-octylphenol and bisphenol A (4,4′-isopropylidenediphenol) were obtained from Aldrich (Milwaukee, WI). Atrazine, was from Chem-Service (West Chester, PA).

Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA), and restriction enzymes were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). Phenol red-free Dulbecco's modified Eagle's medium (DMEM) and medium supplements were from Life Technologies (Gaithesburg, MD). Fetal bovine serum (FBS) and D-luciferin were purchased from Intergen (Purchase, NY) and Molecular Probes (Eugene, OR), respectively. All other chemicals and biochemicals were of the highest quality available from commercial sources.

Construction of plasmids

The plasmid pG4M-hERαdef (Gal4-HEG0) was a gift from Dr. P. Chambon (IGBMC CNRS-LGME, Illkirch Cedex C.U. de Strasbourg, France). The construction of pG4M-mERβdef has been described elsewhere (18). The plasmid pG4M-rtERαdef was constructed by PCR amplifying amino acid residues 214-576 of the rainbow trout ERα as described previously (Matthews et al. in press). The plasmid pG4M-mERαdef (a.a. 268-599) was generated by PCR amplification of the plasmid pJ3MOR containing the complete mouse ER cDNA (provided by M.G. Parker, Molecular Endocrinology Research Laboratory, London, United Kingdom) using primers 5′-caaagaaattcatcgattggcggcatacggaaagaccgc-3′ (forward) and 5′-aaaagaattcgcggccgctcagatcgtgttggggaagccctc-3′ (reverse). The plasmid pG4M-cERdef (a.a. 257-599) was constructed by PCR amplification using primers 5'-aaaactcgagccaaaggtggaatccggaaagac-3′ (forward) and 5′-aaaaagatctttatattgtattctgcatactctcctct-3' (reverse). The plasmid pG4M-aERdef (a.a. 8-349) was generated by PCR amplification using primers 5'-aaaa ggatccctcgagccggtggaattcggaaagaccgcag-3' (forward) and 5'-aaaaggatccctcgagtcaaattgcttcctgctcatttccc-3' (reverse), whereas the plasmid pG4M-xERdef (a.a. 248-586) was constructed by PCR amplification of the plasmid CMV5xER1 containing the complete Xenopus ER cDNA (provided by Dr. D. Shapiro, University of Chicago, Urbana, IL) using primers 5′-aaaactcgagccgggggcattcgaaaggatcgca-3' (forward) and 5′-aaaaggtaccgagctctcatactgtgctttgtaagctcact-3' (reverse). PCR fragments were digested with the appropriate restriction enzymes and ligated into the eukaryotic

expression vector containing the DNA binding domain of the yeast transcription factor Gal4, pG4MpolyII (Dr. P. Chambon). The plasmid pGEXKG-ACTR (SRC-3) (a.a. 615 to 768) was a gift from Dr. L. Freedman (Memorial Sloan-Kittering Cancer Center, New York, NY), whereas the plasmid pGEX-TIF2 (SRC-2) (a.a. 594 to 766) was constructed by PCR amplification of the plasmid pSG5-TIF2 (Dr. P. Chambon) using primers 5'-caaaggatccgaaggtacaactggacaagcagag 3' (forward) and 5'-caaactcgagtcaatctgcttactgtccagtctctc 3' (reverse). PCR fragments were digested with the appropriate restriction enzymes and ligated into pGEX6p3, a GST expression vector (Amersham Pharmacia, Piscataway, NJ).

All PCR amplification was performed as previously described (19). The sequence of each construct was confirmed with restriction enzyme digest and ABI/Prism automated sequencing (Perkin Elmer Applied Biosystems, Foster City, CA).

Cell culture and transient transfection assays

MCF-7 human breast cancer estrogen receptor positive cells (obtained from Dr. L. Murphy, University of Manitoba) were maintained with phenol red-free DMEM supplemented with 3.7 g/l $NaHCO_3$, 2 mM L-glutamine, 10% FBS, 10 mM HEPES, 500 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. Cells were cultured in a humidified environment at 37°C with 5% CO_2.

Transient transfections and gene transcription assays were performed essentially as previously described (20,21). Briefly, MCF-7 cells were seeded at approximately 50% confluency in 6-well tissue culture plates in medium supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and allowed to settle for 7 h.

MCF-7 cells were transiently transfected by the calcium phosphate precipitation method (22) with 1.5 μg of 17m5-G-Luc (provided by Dr. P Chambon), 0.2 μg of either pG4M-hERαdef, pG4M -mERαdef, pG4M -mERβdef, pG4M -rtERαdef or 0.5 μg of pG4M -cERdef, pG4M -cERdef, or pG4M -xERdef, and 0.05 μg of pCMV-lacZ (β-galactosidase expression vector). Transiently transfected cells were washed 16 h later with sterile phosphate buffered saline and fresh medium was added to each well.

Transiently transfected cells were exposed to final concentrations ranging from 10^{-12} to 10^{-5} M of test compound. Final concentrations were obtained by adding 2 μl of test chemical to 2 ml of medium. After a 24 h incubation with test compound, cells were harvested and assayed for luciferase activity according to standard methods (23).

Each treatment was done in duplicate and two samples were taken from each replicate. Each experiment was repeated three times. Values are reported as a percentage relative to the maximum induction observed with E2.

Reverse transcriptase polymerase chain reaction

Total RNA from MCF-7 cells was isolated using the Trizol reagent according to manufacturer's instructions (Life Technologies). In brief, cells were lysed with two successive washes of 325 μl aliquots of Trizol. The lysates were pooled and incubated with 300 μl of chloroform for 10 min at 23°C. The mixture was separated by centrifugation at 12,000xg for 15 min at 4°C and the aqueous layer (1 ml) containing the RNA was removed and precipitated with 500 μl of isopropanol and 2 μl of linear acrylamide (10 mg/ml). The RNA was pelleted by centrifugation at 12,000xg for 10 min at 4°C and the resulting pellet was washed with 75% ethanol. The pellet was then air

dried and resuspended in 100uL of 3M sodium citrate. The RNA was stored at –20°C until use.

Total RNA (5 μg) was incubated for 10 min at 70°C with 0.5μg oligo dT primer (5'-ttttttttttttttttttttvn-3'). The reaction was chilled on ice for 2 min and the mRNA was reverse transcribed in a 20 μl reaction mixture containing PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), 10 mM DTT, 500 μM dNTPs and 200 IU of SuperScript II reverse transcriptase for 60 min at 42°C. The reaction was terminated with a 15 min incubation at 70°C. One μl of the reverse transcription reaction was used in the subsequent PCR reactions using the following primers for β-actin: 5'-aaaagcatccaagcttctgaagtaccccattgaacatggca-3' (forward) (5'-aaaactcgaggcggccgctgtcacgcacgatttccctctcag-3' (reverse), SRC-1: 5'-caaaccatggatccagacagtaaatactctcaaaccagtc-3' (forward) (5'-caaactcgagtcaatcaggctcgacagacaaagtgg-3' (reverse), SRC-2: 5'-caaaggatccagaaggtacaactggagaagcagag-3' (forward) and 5'-caaagaattctcagtgatggtgatggtgatgatctgtcttactgtccagtctctc-3' (reverse), and SRC-3: 5'-gaaagtaaggagagcagtgttgag-3' (forward) and 5'-gtcagaactagtcagatcaccaag 3' (reverse). After the addition of template, the samples were incubated at 94°C for 3 min and amplified for 25 cycles. Each cycle included: 45 sec. denaturation at 94°C, 45 sec annealing at 60°C and 1 min elongation at 72°C. PCR products were separated by 2% agarose electrophoresis and visualized with ethidium bromide staining.

GST pull-down assays

The expression and purification of GST fusion proteins was performed as described previously (24,25). Glutathione-Sepharose beads (Amersham Pharmacia) were prewashed in NETN buffer (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol). Crude bacterial extracts containing the fusion proteins (GST-SRC-1, GST-SRC-2, and GST-SRC-3) were purified onto the prewashed beads (10 µl beads + 20 µl crude in 500 µl total reaction) by incubation at 4°C for 1.5 h on a rotary mixer. The beads (loaded with fusion protein) were collected by centrifugation and washed three times with NETN buffer. GST fusion proteins or GST alone were incubated overnight in 1.5 ml centrifuge tubes containing 490 µl of NETN buffer containing 1% bovine serum albumin, 5 µl of the *in vitro* translated ^{35}S-labeled receptor, in the presence of 5 µl of vehicle or test compound. Beads were washed four times with NETN, and dried under vacuum for 10 min. The dried beads were resuspended in 25 µl of 3x protein loading buffer (reducing), incubated for 5 min at 95°C, and the entire sample was separated by 12% SDS-PAGE. The gels were fixed, dried, and the radiolabelled rtER was visualized by fluorography. The amount of bound ^{35}S-labelled protein was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

Ability of 17β-estradiol to induce gene expression mediated by ERs from several different species

E2 and 14 EEDs, several of which have been shown to exhibit preferential affinity for different vertebrate ERs (Chapter 4 and reference (24)) were examined for their ability to induce ER-mediated gene expression by measuring luciferase activity using MCF-7 cells co-transfected with the Gal4-ERdef and a Gal4-regulated luciferase reporter gene, 17m5-G-Luc. The amino acid residues of the ER D, E, and F domains used to generate the Gal4-ERdef fusion proteins Gal4-hERαdef (human α), Gal4-mERαdef (mouse α), Gal4-mERβdef (mouse β), Gal4-cERdef (chicken), Gal4-aERdef (anole), Gal4-xERdef (xenopus), and Gal4-rtERαdef (rainbow trout) and their sequence identity compared to hERα are shown in figure 1. The results in figure 2 show that E2 treatment of transiently transfected MCF-7 cells with Gal4-ERdef caused a concentration-dependent increase in luciferase activity. The EC_{50} value for this response was similar among the different chimeric receptors ranging from 0.05 to 0.7 nM. However, the E2 induced response mediated by Gal4-rtERαdef was 2 orders of magnitude lower, with an EC_{50} value of 28 nM when transfected cells were maintained at 37°C. When, MCF-7 cells transiently transfected with Gal4-rtERαdef were incubated at 20°C, a more physiological relevant temperature for rainbow trout, the EC_{50} value was reduced to 1 nM. The molecular basis for this temperature sensitive phenotype of the rtERα is more thoroughly investigated in chapter 6. Surprisingly, neither the xenopus nor

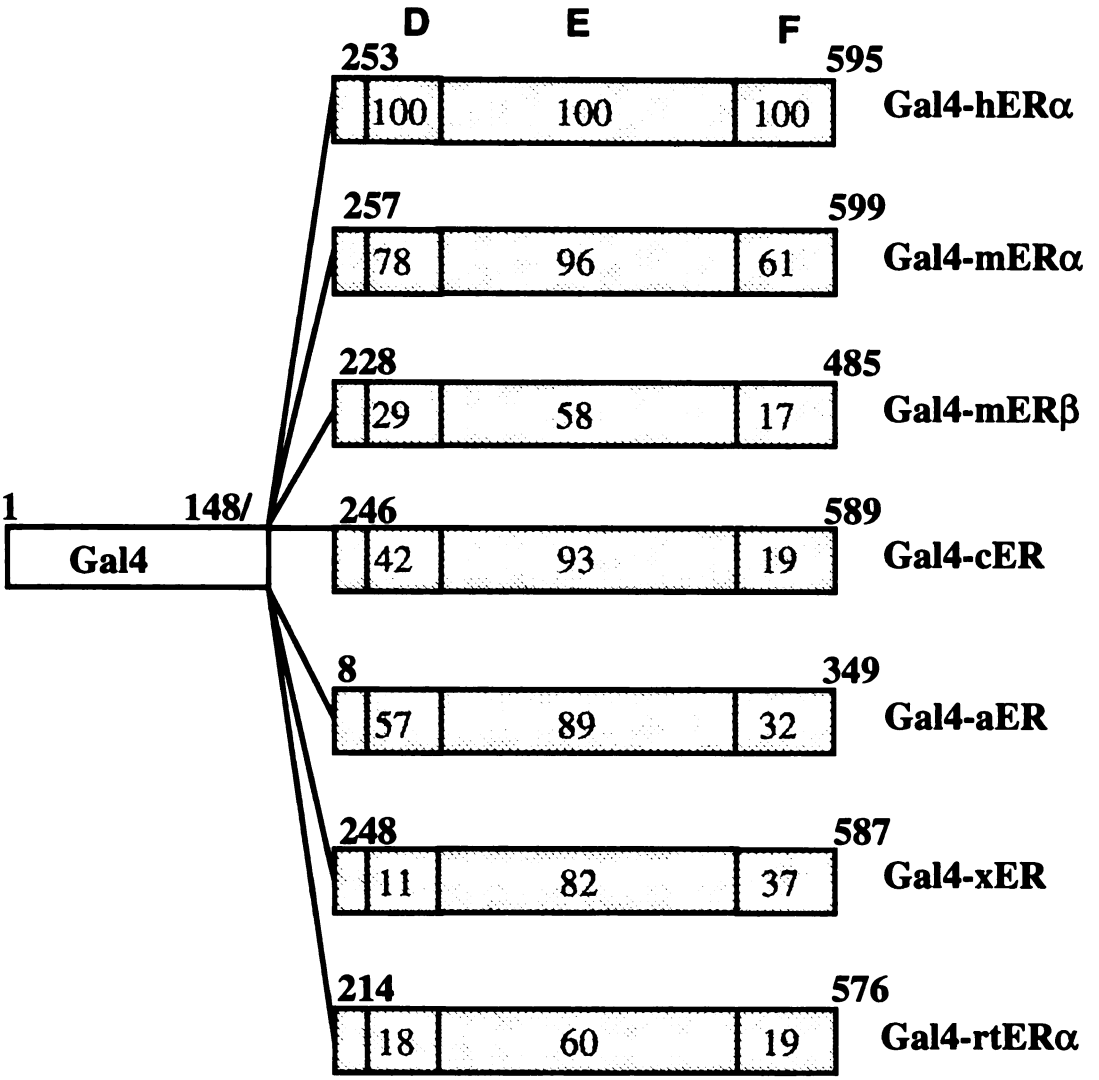

Figure 1. Schematic representation of Gal4-ER chimeric receptors transiently transfected into MCF-7 human breast cancer cells. The chimeric receptors consisted of the DNA binding domain of the yeast transcription factor, Gal4, linked upstream of the last 11 amino acid residues of the C domain and the entire D, E, and F domains of ERs from several vertebrate species. Numbers provided above identify the amino acids used in the construction of the expression vectors. The numbers wthin the domains indicate the % amino acid sequence identity compared to hERα.

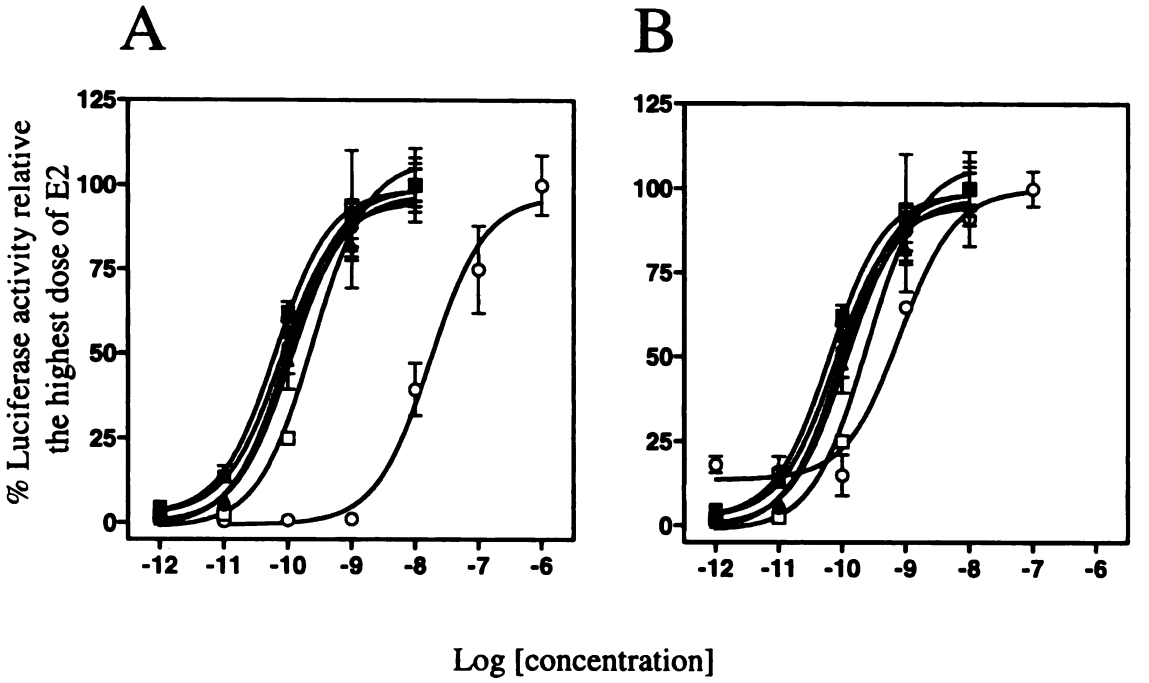

Figure 2. Comparison of the ER-induced transactivation ability mediated by Gal4-ERs. MCF-7 human breast cancer cells transiently transfected with 0.2 μg Gal4-hERα (●), Gal4-mERβ (■), Gal4-rtERα (○) or 0.5 μg of Gal4-cER (▲), Gal4-aER (◆), Gal4-xER (□), 1.5 μg of Gal4 regulated-luciferase reporter gene, 17m5-G-Luc, and pCMV as described in the methods section. Following dosing with E2, cells were incubated at the 37°C (panel A), with the exception of cells transfected with Gal4-rtERα which were incubated at 20°C (panel B).

the anole ERs exhibited temperature sensitive phenotypes as judged by their ability to induce reporter gene expression at 37°C (Figure 2).

The maximal E2 induction ranged from 20- to 100-fold among the Gal4-ERs. Due to the variability in fold induction the data are reported as percent luciferase activity relative to the maximal activity achieved by E2 with each receptor, which was arbitrarily set to 100% for comparative purposes.

RtERα recruits SRC coactivators

GST pull-down assays were performed to verify that the reduced transactivation activity of the rtERα was not due to an inability of the rtERα to interact with human coactivators. Figure 3 shows a comparison of the expression levels of three different classes of the SRC family of coactivators. RT-PCR analysis of total RNA isolated from MCF-7 cells revealed that the expression level of SRC-3 (ACTR) was approximately 5-fold higher than either SRC-1 or SRC-2 (TIF2). Figure 4 shows a typical GST pull-down assay, where the dose-dependent recruitment of ^{35}S-rtERα by GST-SRC-2 and GST-SRC-3 with E2 is demonstrated. Similar results were seen with SRC-1 (data not shown). These data suggest that differences in transactivation are not a result of the rtERα failing to recruit human coactivators and are rather due to functional differences between the hERα and rtERα that may be the result of the sequence variability within their respective ligand binding domains.

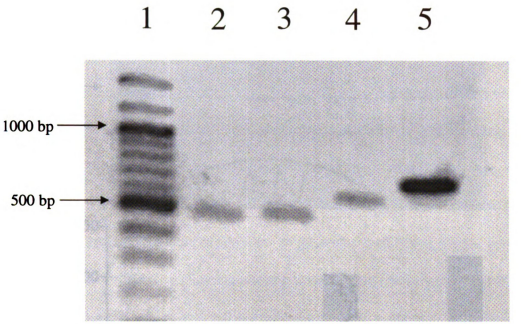

Figure 3. Differential expression levels of SRC coactivators in MCF-7 cells. Total RNA from MCF-7 cells was reverse transcribed with an oligo dT primer, followed by a 25 cycle PCR amplification with gene specific primers. The samples were separted on 2% agarose gel and visualized with ethidium bromide staining. Lane 1: 100 bp molecular marker, lane 2: β-actin , lane 3: SRC-1, lane 4: SRC-2, and lane 5: SRC-3.

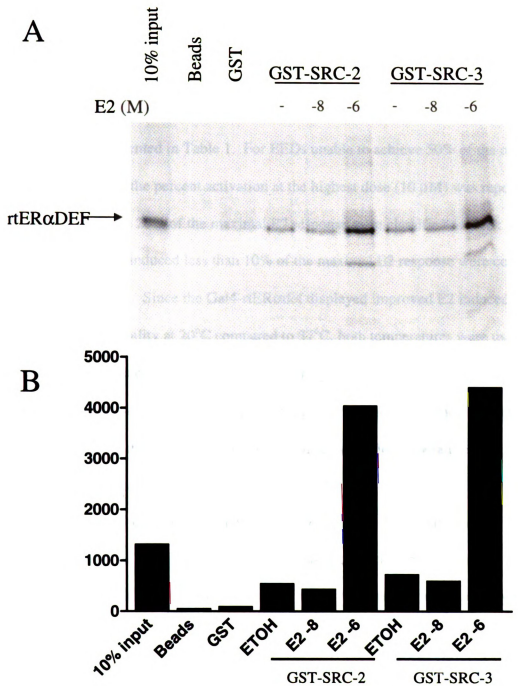

Figure 4. The rtERα DEF binds human SRC-2 and SRC-3. (A) GST fusion proteins previously coupled to Sepharose beads were incubated with in vitro translated, [^{35}S]methionine-labelled rtERα DEF, in the presence or absence of 17β-estradiol (E2) After extensive washing, samples were separated by 12% SDS-polyacrylamide gels. Gels were fixed, dried and visualized by fluorography. (B) Graph of the quantification of the pixel intensity using a phosphorimager.

Ability of EEDs to induce gene expression mediated by ERs from several different species

A summary of the half maximal activation (EC_{50}) values for the 14 EEDs examined is presented in Table 1. For EEDs unable to achieve 50% of the maximal activation of E2, the percent activation at the highest dose (10 μM) was reported. EEDs unable to achieve 20% of the maximal E2 response were classified as weak inducers (wi), while EEDs that induced less than 10% of the maximal E2 response were considered non-inducers (ni). Since the Gal4-rtERαdef displayed improved E2 induced transactivation ability at 20°C compared to 37°C, both temperatures were used to examine rtERα mediated gene expression. In general, the ability of several compounds to induced Gal4-rtERdef-mediated reporter gene expression was increased at 20°C (Table 1) and therefore this was considered to be a more suitable temperature to examine rtERα mediated transactivation activity.

Overall the E2 induced reporter gene expression was similar among the ERs, though several differences in the rank order and transactivation response of EEDs were observed. DES and E2 exhibited a similar ability to induce reporter gene expression mediated by the different ERs, whereas α-zearalenol induced Gal4-rtERαdef-mediated reporter gene expression at lower concentrations than E2 or DES, which was not observed in other Gal4-ERs. The rank order of the phytoestrogens was similar among the ERs (coumestrol >/= genistein>>naringenin), though differences in potency among the receptors were observed. Coumestrol induced reported gene expression 164- and 8-fold greater mediated through Gal4-mERβdef and Gal4-aERdef, respectively, than through

Table 1. EC_{50} values of luciferase reporter gene activity mediated by the ERs from several different species following a 24 h exposure to natural and synthetic estrogenic chemicals at 37°C, unless stated otherwise.

	EC_{50} (M)[a]							
	Gal4-hERα	Gal4-mERα	Gal4-mERβ	Gal4-cER	Gal4-aER	Gal4-xER	Gal4-rtERα 37°C	Gal4-rtERα 20°C
17β-estradiol	$9.5 \pm 9.2 \times 10^{-11}$	$6.2 \pm 3.5 \times 10^{-11}$	$7.9 \pm 2.7 \times 10^{-11}$	$2.8 \pm 0.8 \times 10^{-10}$	$4.5 \pm 2.6 \times 10^{-11}$	$4.9 \pm 8.4 \times 10^{-10}$	$2.8 \pm 0.8 \times 10^{-8}$	$1.0 \pm 0.5 \times 10^{-9}$
DES	$1.8 \pm 0.7 \times 10^{-10}$	$3.2 \pm 2.6 \times 10^{-11}$	$6.6 \pm 1.2 \times 10^{-11}$	$1.9 \pm 1.0 \times 10^{-10}$	$4.9 \pm 3.8 \times 10^{-11}$	$1.8 \pm 0.8 \times 10^{-10}$	$1.5 \pm 0.8 \times 10^{-8}$	$9.3 \pm 4.3 \times 10^{-10}$
α-Zearalenol	$7.8 \pm 3.2 \times 10^{-10}$	$1.0 \pm 0.1 \times 10^{-10}$	$4.4 \pm 0.6 \times 10^{-10}$	$9.6 \pm 3.3 \times 10^{-10}$	$5.6 \pm 3.2 \times 10^{-9}$	$2.5 \pm 2.1 \times 10^{-9}$	$2.2 \pm 1.2 \times 10^{-8}$	$2.1 \pm 0.7 \times 10^{-10}$
β-Zearalenol	$7.0 \pm 2.6 \times 10^{-9}$	$1.8 \pm 1.5 \times 10^{-9}$	$2.2 \pm 1.0 \times 10^{-9}$	$3.3 \pm 0.5 \times 10^{-8}$	$5.3 \pm 2.7 \times 10^{-8}$	$7.5 \pm 4.0 \times 10^{-9}$	$6.0 \pm 2.9 \times 10^{-8}$	$9.8 \pm 0.1 \times 10^{-9}$
DHT	$3.8 \pm 3.0 \times 10^{-8}$	3.0×10^{-8}	$1.9 \pm 0.9 \times 10^{-8}$	$1.0 \pm 1.4 \times 10^{-7}$	$2.1 \pm 0.7 \times 10^{-8}$	$4.4 \pm 1.1 \times 10^{-7}$	16%	$1.0 \pm 0.7 \times 10^{-7}$
BPA	$2.7 \pm 0.2 \times 10^{-6}$	2.1×10^{-7}	$3.9 \pm 1.2 \times 10^{-7}$	25%	30%	30%	50%	$3.3 \pm 2.6 \times 10^{-7}$
4-*t*-octylphenol	$1.0 \pm 1.5 \times 10^{-7}$	7.3×10^{-8}	$2.5 \pm 0.4 \times 10^{-8}$	$1.2 \pm 1.9 \times 10^{-7}$	$4.5 \pm 1.8 \times 10^{-8}$	30%	20%	$7.1 \pm 3.8 \times 10^{-7}$
Coumestrol	$6.1 \pm 3.7 \times 10^{-7}$	5.7×10^{-7}	$3.7 \pm 1.8 \times 10^{-9}$	$6.3 \pm 5.1 \times 10^{-7}$	$7.5 \pm 0.6 \times 10^{-8}$	$7.2 \pm 2.8 \times 10^{-7}$	50%	$5.1 \pm 2.1 \times 10^{-7}$
Genistein	$4.0 \pm 4.2 \times 10^{-7}$	1.8×10^{-7}	$4.2 \pm 0.6 \times 10^{-9}$	$2.0 \pm 1.1 \times 10^{-7}$	$1.5 \pm 0.7 \times 10^{-7}$	$9.8 \pm 5.9 \times 10^{-7}$	$3.3 \pm 0.3 \times 10^{-6}$	$2.3 \pm 1.4 \times 10^{-7}$
Naringenin	$3.0 \pm 0.2 \times 10^{-5}$	2.0×10^{-6}	$4.1 \pm 0.8 \times 10^{-7}$	$1.6 \pm 0.8 \times 10^{-5}$	$2.1 \pm 1.6 \times 10^{-6}$	ni	19%	$4.2 \pm 2.5 \times 10^{-6}$
Methoxychlor	wi[b]	wi	wi	ni	ni	ni	ni	ni
o,p` DDT	50%[c]	1.1×10^{-6}	ni	$8.0 \pm 0.8 \times 10^{-7}$	ni	ni	ni	ni
p,p`DDT	wi	nd[e]	ni	ni	ni	ni	ni	ni
β-Sitosterol	ni[d]	nd	ni	ni	ni	ni	ni	ni
Atrazine	ni	nd	ni	ni	ni	ni	ni	ni

[a] EC_{50} values were determined using the sigmoidal dose-response function in GraphPad 3.0.

[b] Denotes weak inducer (wi) since treatment resulted in less than a 20% increase in reporter gene activity

[c] Refers to percent luciferase activity at the highest dose examined (10 μM).

[d] Denotes non inducer (ni) since no significant induction of reporter gene activity was observed at the highest examined dose (10 μM)

the other receptors. Genistein and naringenin also preferentially induced reporter gene expression mediated by Gal4-mERβdef at concentrations 70 to 95 fold lower than the other receptors (Figure 5). Although these phytoestrogens are less potent than E2, coumestrol and genistein were able to generate a response mediated through ERβ at concentrations similar to physiological hormone levels (10-100 nM). β-Sitosterol did not induce any significant reporter gene expression through any of the ERs examined.

Of the industrial chemicals examined, 4-t-octylphenol was the most potent among the different ERs with EC_{50} values ranging from 0.01 to 0.1 μM (Figure 6). The EC_{50} values for bisphenol A (BPA) ranged from 0.3 to 3 μM, with BPA exhibiting a preferential ability to induce reporter gene expression mediated by Gal4-mERβdef and Gal4-rtERαdef. BPA was only able to induce 25-30% of maximal activity of E2 through Gal4-cERdef, -aERdef, and -xERdef. The pesticide o,p′-DDT exhibited the most variability of all the compounds examined. o,p′-DDT induced reporter gene expression with EC_{50} values of 0.8 ± 0.1 and 2.9 ± 2.5 μM for Gal4-cERdef and Gal4-mERαdef, respectively. However, only 40% maximal response was observed following incubation with cells transiently transfected with Gal4-hERαdef and o,p′-DDT was classified as a non-inducer for the other receptors (Figure 6). Methoxychlor, p,p′-DDT, and atrazine failed to significantly induce reporter gene expression through any of the ERs. These data show that certain estrogenic compounds exhibit a differential ability to induce reporter gene activity mediated by both ER isoforms and ERs from different vertebrate species. Although differences in the ability of EEDs to induce gene expression were observed among the different vertebrate ERs, the most dramatic differences were observed with the phytoestrogens interacting with ERβ.

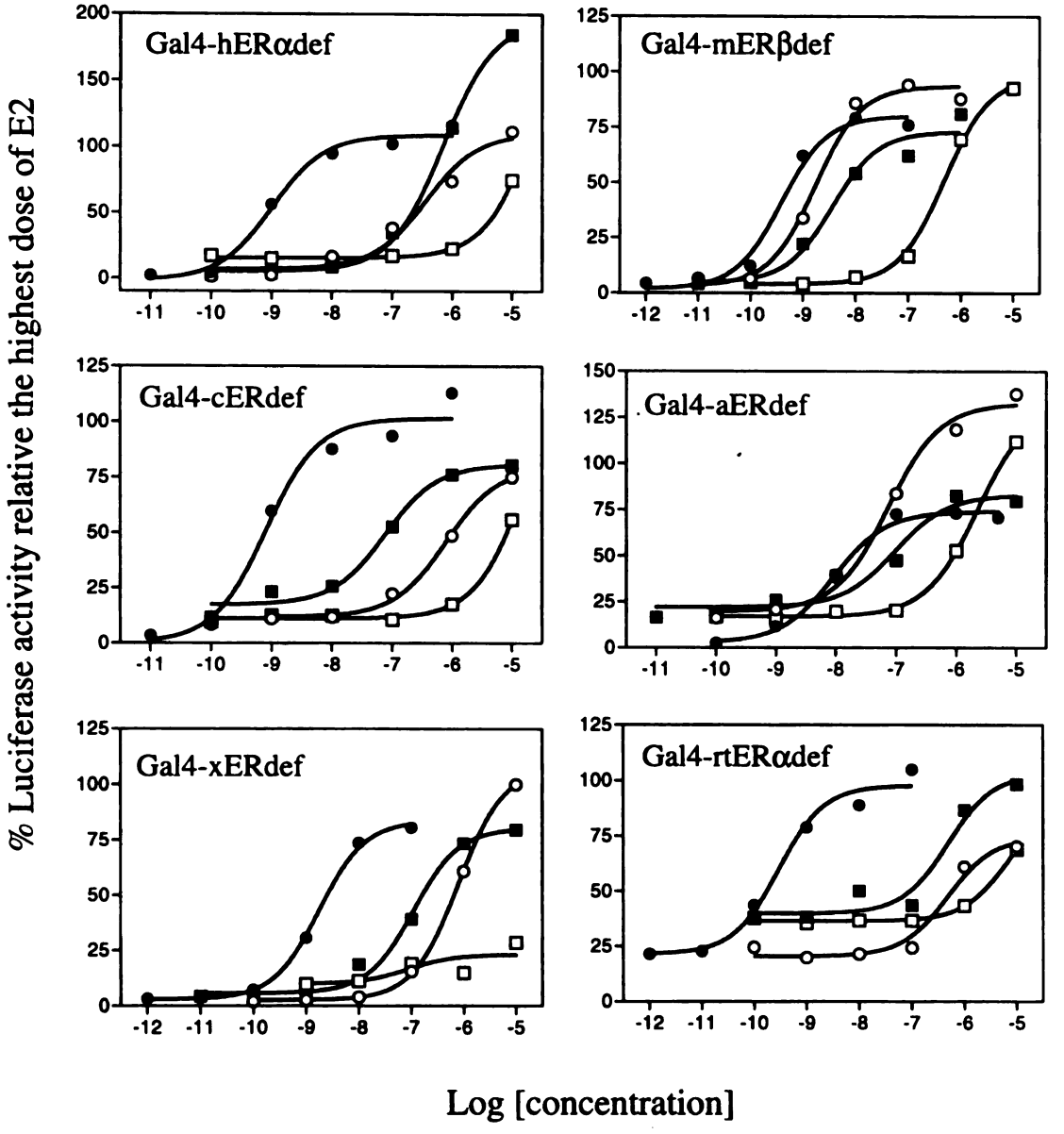

Figure 5. Mycotoxins and phytoestrogens differ in their ability to induce gene expression mediated by both ER isoforms and ERs from different vertebrate species. Comparison of the ability of α-zearalenol (●), coumestrol (■), genistein (○), and naringenin (□) to induce gene expression mediated by Gal4-hERαdef, Gal4-mERβdef, Gal4-cERdef, Gal4-aERdef, Gal4-xERdef, or Gal4-rtERαdef. MCF-7 human breast cancer cells were transiently transfected with 0.2 to 0.5 μg of Gal4-ER chimeras, 1.5 μg of 17m5-G-Luc, and 0.1 μg of pCMV as described in the methods section. Cells transfected with Gal4-rtER were incubated for 24 h at 20°C after dosing with test compound. Cells transfected with the other Gal4-ER chimeric receptors were incubated for 24 h at 37°C.

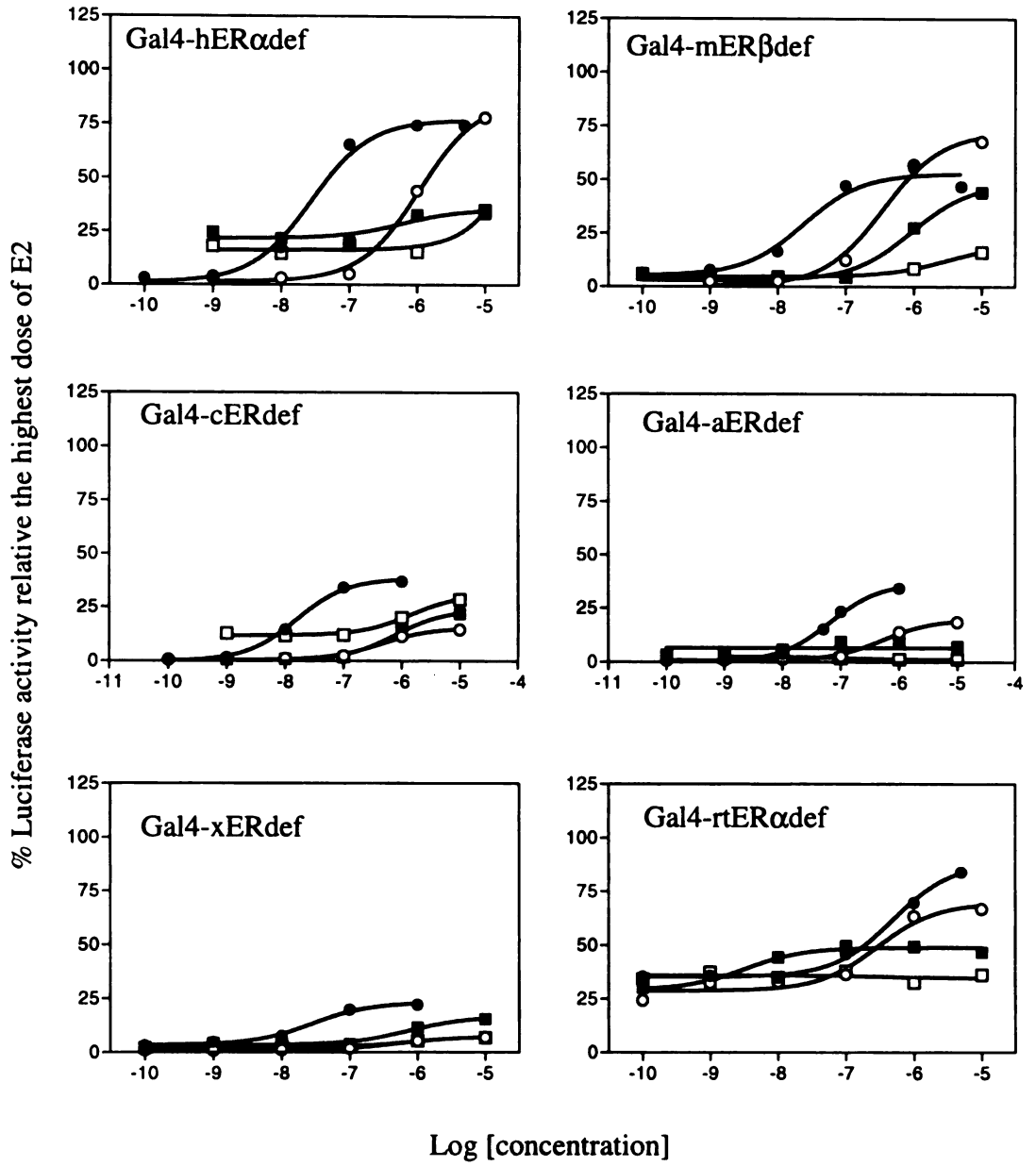

Figure 6. Xenobiotics differ in their ability to induce gene expression mediated by both ER isoforms and ERs from different vertebrate species. Comparison of the ability of 4-*t*-octylphenol (●), Bisphenol A (■), *o,p´*-DDT (○), and methoxychlor ( ) to induce gene expression mediated by Gal4-hERαdef, Gal4-mERβdef, Gal4-cERdef, Gal4-aERdef, Gal4-xERdef, or Gal4-rtERαdef. MCF-7 human breast cancer cells transiently transfected with Gal4-ER chimeras described in the methods section. Cells transfected with Gal4-rtERα were incubated for 24 h at 20°C after dosing with test compound. Cells transfected with the other Gal4-ER chimeric receptors were incubated for 24 h at 37°C.

Discussion

In chapter 4, ligand binding assays were used to examine the ability of several EEDs to bind to hERα, mERα, cER, aER, and rtERα. Several differences in relative and absolute binding affinities were observed, with the rtERα, which has the greatest amino acid variation in its ligand binding domain relative to the other species examined, exhibiting the most striking differences. In radioligand binding assays only compounds capable of competing with radioligand are detected. Also, these assays do not distinguish between agonists and antagonists. Cell based transcription assays using a reporter gene under the transcriptional control of a specific receptor offer an alternative to radioligand binding assays and provide valuable information about the ability of test compounds to induce transcriptional responses. In order to focus on differences in transactivation ability among different vertebrate ERs that may reside within their respective ligand binding domains, Gal4-ERdef fusion proteins consisting of the D, E, and F domains of each receptor were constructed. This ensured that effects imposed by other domains, such as the DNA binding domain would not influence the results (26) and also allowed for comparison with the competitive binding data presented in chapter 4 where similar GST-ERdef fusion proteins were used. The Gal4-ERdef fusion proteins exhibited similar E2 transactivation ability when compared to full-length ERs (27,28). Although the rtERα exhibited reduced transactivation ability at 37°C, once the incubation temperature was reduced to 20°C, the E2-induced transactivation response agreed with previous studies in yeast transiently transfected with full-length rtERα (29). This is consistent with another study investigating the transactivation ability of the Oreochromis aureus

ERα (OaERα), which also exhibits reduced activity at temperatures above its normal physiological range (30).

There were instances where the ligand binding data did not support the gene expression results. For example, 4-t-octylphenol preferentially bound to GST-rtERαdef with 10-fold greater affinity than to the GST-aERdef, but was able to induce reporter gene expression at approximately 10-fold lower concentrations through aER than through rtERα. In addition, some compounds found to compete for binding to a specific ER were unable to induced gene expression mediated by the ER from the same species. For example, o,p'-DDT bound to GST-rtERαdef but did not elicit any reporter gene activity mediated by Gal4-rtERαdef.

In many other instances the gene expression data support the competitive binding studies presented in chapter 4. The mycotoxin, α-zearalenol, which bound with greater affinity than E2 to GST-rtERαdef, also induced reporter gene expression mediated by Gal4-rtERαdef at lower concentrations than E2. Similarly, coumestrol which bound to GST-aERdef with higher affinity than to the other ERs, also induced gene expression mediated by Gal4-aERdef at lower concentrations compared to the Gal4-hERαdef, -mERαdef, -cERdef, and -rtERαdef. A plot of the log transformed IC_{50} values (Chapter 4, table 2, page 77) vs EC_{50} values (Table 1 page 101) for hERα, mERα, cER, aER, and rtERα shown in figure 7 reveals a linear correlation with a Pearson r value of 0.86. Linear regression analysis revealed an overall slope of 0.82, indicating a strong correlation between ligand binding and transactivation ability.

The half-maximal responses of E2 and DES in the gene expression assay were approximately 10-fold lower than that of the ligand binding assay. In contrast the half-

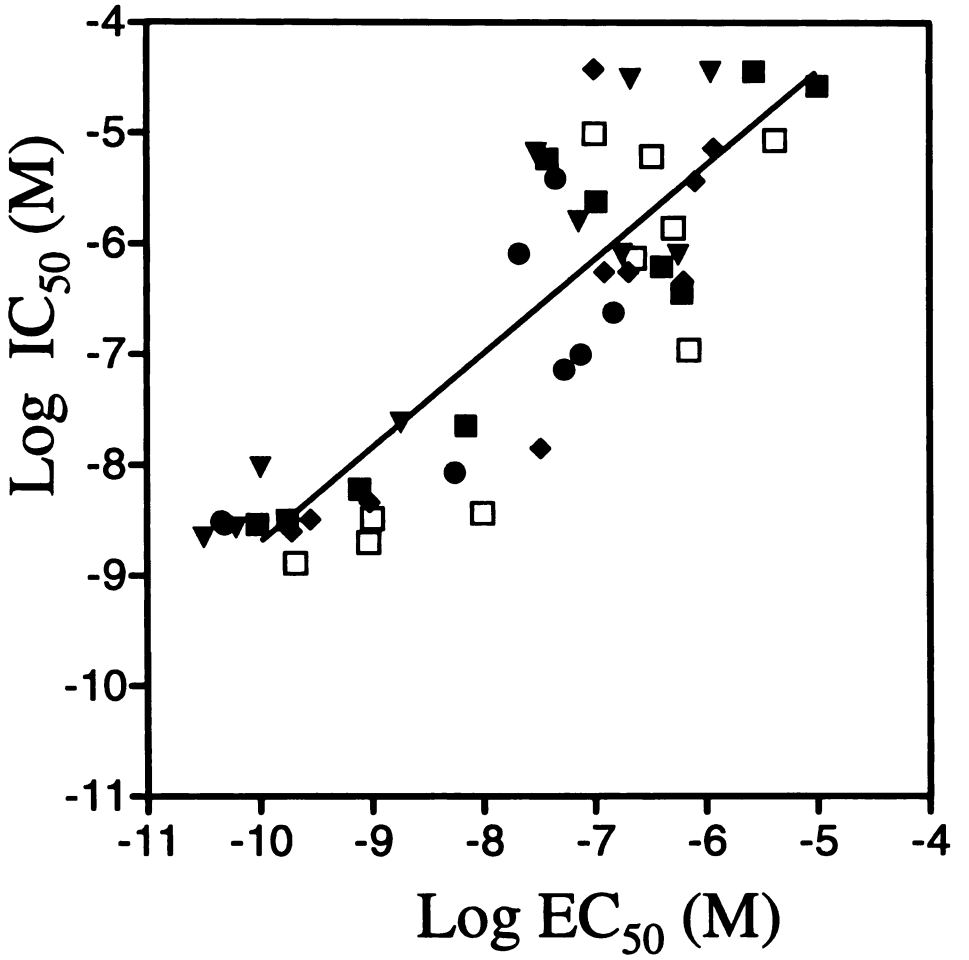

Figure 7. Comparison of IC_{50} and EC_{50} values among different vertebrate ERs. Correlation analysis of the log transformed IC_{50} values (Chapter 4, Table 2, page 76) vs EC_{50} values (Table 1, page 101) for hERα(●), mERα (○), cER (■), aER (□), and rtERα (◆) reveals an overall linear correlation with a Pearson r value of 0.86.

maximal responses to ligand for the progesterone receptor (PR) and the glucocorticoid receptor (GR) reflect their affinity for that ligand (31,32). Unlike the GR and PR, the ER is capable of binding DNA in the absence of ligand (33) which may be a contributing factor to the hormone-independent activity of the receptor and as been proposed has an explanation for the discrepancy in half-maximal response between gene expression and ligand binding assays (27). DNA binding and subsequent recruitment of coactivators may represent a rate limiting process in the activation of NRs. Since the ER can exist in a "prebound state" to DNA, essentially overcoming the rate-limiting step, maximal transactivational activity may be achieved at lower concentrations (27). Gel shift assays using the Gal4-ER constructs have not been performed to support this hypothesis.

The phytoestrogens exhibited an increased ability to induce gene expression mediated by Gal4-mERβdef, which was consistent with previous competitive binding studies where genistein exhibited a 30-fold greater affinity for ERβ compared to ERα (1). These previous studies reported only a 3-fold lower EC_{50} value for ERβ compared to ERα (1). This is far lower than the 95-fold (genistein) and 164-fold (coumestrol) lower EC_{50} values for mERβ compared to hERα reported in the present study (Table 1). These discrepancies may be due to differences in the cell–based assay conditions, since the previous studies were done in human embryonic kidney 293 cells transiently transfected with full length hERα and hERβ and an ERE driven reporter gene (1).

EEDs have also been shown to differentially recruit the coactivators, SRC-1 and SRC-2 to ERα and ERβ, in which both full-length and ligand-binding domain truncations were used (16). The HO-PCB, 2',3',4',5-tetrachlorobiphenyl, exhibited a similar RBA for both ERα and ERβ. Treatment of this compound, however, failed to recruit SRC-1 or

SRC-2 to ERα but resulted in complete recruitment of SRC-1 and submaximal recruitment of SRC-2 to ERβ. The 30-fold greater binding affinity of genistein for ERβ resulted in a 12 000- and 33-fold greater ability of ERβ to recruit SRC-1 and SRC-2, respectively (16), which supports the increased ability of genistein to induce ERβ-mediated reporter gene activity (Table 1, Figure 5). However, x-ray crystallographic data of the genistein-ERβ complex reveals that the critical helix 12, which contains the AF-2 region is positioned intermediate between agonist and antagonist conformations suggesting that the complex may not adequately interact with coactivators. The differential expression levels of coactivators in cell lines combined with the observation that SRC family of coactivators interact with NRs via both the AF-1 and AF-2 regions may also account for some of the discrepancies observed among different studies.

Take together these results demonstrate that ERs from different vertebrate species exhibit differential transactivation profiles in response to a number of EEDs. The ability of E2 and other estrogenic chemicals to induce gene expression mediated by the rtERαdef is influenced by temperature. In general the results from the ligand binding studies presented in chapter 4 support the transactivation data present here, though there were instances where the two assays did not correlate. Pharmacokinetic and pharmacodynamic differences among species make it difficult to predict whether the differences in functional activities among ERs would be observed in vivo.

References

1. Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998) *Endocrinology* **139**, 4252-4263.

2. Fitzpatrik, D. W., Picken, C. A., Murphy, L. C., and Buhr, M. M. (1989) *Comp Biochem Physiol* **94C**, 691-694.

3. Strunck, E., Stemmann, N., Hopert, A., Wunsche, W., Frank, K., and Vollmer, G. (2000) *J Steroid Biochem Mol Biol* **74**, 73-81.

4. Glass, C. K., and Rosenfeld, M. G. (2000) *Genes Dev* **14**, 121-141.

5. Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997) *Curr Opin Cell Biol* **9**, 222-232.

6. Katzenellenbogen, B. S. (1996) *Biol Reprod* **54**, 287-293.

7. Onate, S. A., Boonyaratanakornkit, V., Spencer, T.E., Tsai, S.Y., Tsai, M.J., Edwards, D.P., O'Malley, B.W. (1998) *J Biol Chem* **273**, 12101-12108.

8. Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., Brown, M. (1994) *Science* **264**, 1455-1458.

9. Hong, H., Kohli, K., Garabedian, M., and Stallcup, M. (1997) *Mol Cell Biol* **17**, 2735-2744.

10. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) *EMBO J.* **1996**, 3667-3675.

11. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) *Science* **277**, 965-968.

12. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90**, 569-580.

13. Takeshita, A., Cardona, G. R., Koibuchi, N., Suen, C. S., and Chin, W. W. (1997) *J Biol Chem* **272**, 27629-27634.

14. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**, 677-684.

15. Li, H., Gomes, P. J., and Chen, J. D. (1997) *Proc Natl Acad Sci U S A* **94**, 8479-8484.

16. Routledge, E. J., White, R., Parker, M. G., and Sumpter, J. P. (2000) *J Biol Chem* **275**, 35986-35993.

17. Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J., and Carlquist, M. (1999) *Embo J* **18**, 4608-4618.

18. Fertuck, K. C., Matthews, J. B., and Zacharewski, T. R. (2001) *Toxicol Sci* **59**, 231-240.

19. Gillesby, B., and Zacharewski, T. R. (1999) *Breast Cancer Res Treat* **56**, 253-265
20. Balaguer, P., Joyeux, A., Denison, M.S., Vincent,R., Gillesby, B.E., Zacharewski, T. (1996) *Can J Physiol Pharmacol* **74**, 216-222.

21. Zacharewski, T. R., Meek, M.D., Clemons, J.H., Wu, Z.F., Fielden, M.F., Matthews, J.B. (1998) *Toxicol Sci* **46**, 282-293.

22. Sambrook, J., Fristch, E.F., Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual.*, Cold Spring Harbor Laboratory Press, New York.

23. Brasier, A. R., Tate, J.E., Habener, J.F. (1989) *Biotechniques* **7**, 1116-1122
24. Matthews, J. B., Celius, T., Halgren, R., and Zacharewski, T. R. (2000) *J Steroid Biochem Mol Bio* **74**, 223-234.

25. Matthews, J. B., and Zacharewski, T. R. (2000) *Toxicol Sci* **53**, 326-339.

26. Petit, F. G., Valotaire, Y., and Pakdel, F. (2000) *Nucleic Acids Res* **28**, 2634-2642.

27. Reese, J. C., and Katzenellenbogen, B. S. (1992) *J Biol Chem* **267**, 9868-9873.

28. Pakdel, F., Le Gac, F., Le Goff, P., and Valotaire, Y. (1990) *Mol Cell Endocrinol* **71**, 195-204.

29. Petit, F., Valotaire, Y., and Pakdel, F. (1995) *Eur J Biochem* **233**, 584-592.

30. Tan, N. S., Frecer, V., Lam, T. J., and Ding, J. L. (1999) *Biochim Biophys Acta* **1452**, 103-120.

31. Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) *Cell* **46**, 645-652.

32. el-Ashry, D., Onate, S. A., Nordeen, S. K., and Edwards, D. P. (1989) *Mol Endocrinol* **3**, 1545-1558.

33. Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I., and Chambon, P. (1989) *EMBO J* **8**, 1981-1986.

CHAPTER 6

Reciprocal mutagenesis between human α (L349, M528) and rainbow trout α (M317, I496) estrogen receptor residues demonstrates their importance in ligand binding and gene expression at different temperatures

These data have been published in the following article:

Matthews, J.B., Clemons, J.H., and Zacharewski, T.R. (2001) Reciprocal mutagenesis between human α (L349, M528) and rainbow trout (M317, I496) estrogen receptor residues demonstrates their importance in ligand binding and gene expression at different temperatures. *Mol Cell Endocrinol* in press

Abstract

Several fish proteins exhibit compromised function at temperatures outside of their normal physiological range. In this study, the effect of temperature on the ligand binding and the transactivation abilities of the rainbow trout estrogen receptor (rtER) and human estrogen receptor alpha (hERα) were examined. Saturation analysis and gene expression assays, using GST-ER and Gal4-ER fusion proteins consisting of the D, E and F domains of human (hERαdef) and rainbow trout (rtERdef) receptors, show that GST-rtERdef E2 binding affinity and transactivation ability decrease with increasing temperature. A comparison of the amino acid sequence differences between their ligand binding pockets identified two conservative amino acid residue substitutions in rtER (M317, I496) and hERα (L349, M528). The effect of these substitutions on ligand binding and transactivation were examined by constructing reciprocal mutants, which effectively exchanged the binding pockets between rtER and hERα. The rtERdef M317L:I496M double mutant exhibited increased E2 binding affinity and transactivation ability at higher temperatures, and displayed hERα phenotypic behavior for the phytoestrogen, coumestrol. The hERαdef L349M:M528I double mutant also exhibited a modest trend towards adopting the rtER phenotype. These studies demonstrate that conservative changes in residue hydrophobicity and volume can significantly affect ER ligand binding and transactivation ability in a temperature-dependent manner. The lack of a complete exchange of phenotypes between rtER and hERα indicates that factors outside of the ligand-binding pocket are also involved.

Introduction

The estrogen receptor (ER) is a member of the nuclear receptor superfamily, a family of ligand-regulated transcription factors (1-3). As with other members of the nuclear receptor superfamily, the ER consists of several functional domains (A-F), with the DNA-binding domain (C domain) separating the NH_2-terminal ligand-independent activation domain (A/B domains) and the COOH-terminal region, which includes a hinge region (D domain), the ligand binding domain (E domain) and a variable F domain (1,4). Two ER subtypes exist, ERα and ERβ, each is encoded from a distinct gene and display tissue-specific distribution and ligand preference (5,6). Although the mechanism of action of estrogen and the domain organization of the ER is well conserved among species, several studies have reported significant differences in transactivation ability and ligand preference, especially between human and rainbow trout (*Oncorhynchus mykiss*) ERs (7-10).

A comparison of the human and rainbow trout ERs indicates that amino acid sequence identity within domains can vary significantly between the two species. The most conserved region is the DNA-binding domain (92%). The NH_2-terminal A/B domains are only 20% identical, while the E domain, which contains the hormone-binding pocket, is 60% identical in amino acid sequence (11,12). Despite the significant sequence identity in some domains, functional differences in response to 17β-estradiol (E2) and other estrogenic chemicals have been reported. For example, when expressed in yeast the rtER exhibits a 10-fold lower responsiveness to E2 and a significantly reduced affinity for E2 at elevated temperature when compared to hERα (10). Weaker E2 responsiveness of rtER has also been observed in embryonic salmonid cells (STE-137)

transfected with rtER (9). In addition, functional differences in response to other hormones and antihormones have been reported (10). The molecular basis for the reduced rtER function at elevated temperature is unclear, although other fish proteins have been shown to exhibit optimal activity at lower temperatures when compared to their mammalian orthologs (13-15).

Domain interchange and the generation of chimeric receptors demonstrated that the rtER (hERα C domain) chimeric receptor had greater DNA binding affinity, while the rtER (hER E domain) chimeric receptor exhibited an increased E2 responsive compared to wild-type rtER (16). These results suggest that some of the functional differences between hERα and rtER reside within the DNA-binding domain. However, competitive binding studies using GST-hERα and GST-rtER fusion proteins consisting of only the D, E and F domains have also identified several differences in relative binding affinities of estrogenic chemicals, supporting the hypothesis that the ligand binding domain significantly contributes to the reported differences between hERα and rtER (8).

Sequence alignment using crystallographic data of the hERα-E2 complex identified two residues (17), L349 and M528 in hERα and the corresponding residues, M317 and I496 in rtER, that differ between their respective ligand binding pockets. In the present study we examined the role these residues play in ligand binding and transactivation by preparing reciprocal mutants, i.e. hERα (L349M, M528I) and rtER (M317L, I496M), which effectively exchanged the ligand binding pockets of hERα and rtER. Our results show that conservative amino acid residue substitutions within the ligand-binding pocket significantly affect ligand binding and transactivation ability. In addition, these residues also contribute to the reduced binding affinity and transactivation

of the rtER at higher temperatures. Interestingly, reciprocal mutagenesis did not result in a complete exchange of phenotypes, suggesting that residues outside of the ligand-binding pocket also influence ligand binding and transactivation ability.

Materials and Methods

Chemicals

17β-estradiol (1,3,5[10]-estratriene-3,17β-diol) and α-zearalenol (2,4-dihydroxy-6-[6α,10-dihydroxy-undecyl]benzoic acid μ-lactone) were purchased from Sigma (St. Louis, MO). Coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Acros Organics (Pittsburgh, PA). 4-*t*-octylphenol, was purchased from Aldrich (Milwaukee, WI). Radiolabeled [2,4,6,7,16,17-^{3}H] 17β-estradiol ([^{3}H]E2; 123 Ci/mmol) was from New England Nuclear (Boston, MA). *pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA), and restriction enzymes were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). Phenol red-free Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) and D-luciferin were purchased from Intergen (Purchase, NY) and Molecular Probes (Eugene, OR), respectively. All other chemicals and biochemicals were of the highest quality available from commercial sources.

Plasmid construction

The plasmids pGEX-hERαdef, and pGEX-rtERdef harboring the human and rainbow trout ER D, E, and F domains were constructed as previously described (7). The pG4M-hERαdef (Gal4-HEG0) was a gift from Dr. P Chambon (IGBMC CNRS-LGME, Illkirch Cedex C.U. de Strasbourg, France). The pG4M-rtERdef plasmid was constructed by PCR amplifying amino acid residues 214-576 of the rainbow trout ER. The fragment was digested with HindIII and ClaI and ligated into the similarly digested eukaryotic expression vector containing the DNA binding domain of the yeast transcription factor

Gal4, pG4MpolyII. PCR amplification was performed essentially as previously described (18).

All mutants were prepared by PCR based mutagenesis strategy, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The oligonucleotides used in the mutagenesis were: 5-tactgaccaacatggcagacagg-3and 5-cctgtctgccatgttggtcagta-3 for hERα L349M (changes Leu349 to Met), 5-catctgtacagcataaagtgcaagaac-3 and 5-gttcttgcactttatgctgtacagatg-3 for hERα M528I (changes Met528 to Ile), 5-gctcaccagcctggctgacaagg-3 and 5-ccttgtcagccaggctggtgagc-3 for rtER M317L (changes Met317 to Leu), and 5- cacctttacagcatgaaatgtaagaac-3 and 5-ttcttacatttcatgctgtaaaggtg-3 for rtER I496M (changes Ile496 to Met). The mutated residues are underlined. The following PCR conditions were used for the mutagenesis: 95°C for 5 min followed by 25 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 10 min. The sequence of each construct was confirmed by restriction enzyme digest and ABI/Prism automated sequencing (Perkin Elmer Applied Biosystems; Foster City, CA).

Expression and purification of GST ER fusion protein

Expression and purification of GST-ERdef fusion proteins was performed as previously described (7,8). Briefly, *E. coli* strain BL21 (Amersham/Pharmacia) containing pGEX-ERdef constructs were incubated at 37°C with constant shaking. Cells were induced with 1 mM IPTG at an optical density of 0.8 at 600 nm and grown for 4 h at 30°C. After centrifugation, cell pellets were resuspended in 25 ml of buffer A (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 50 mM NaCl and 10% (v/v) glycerol, pH 7.5) containing 0.1 mg/ml lysozyme, and protease inhibitors. Cells were then lysed by

sonication on ice and cell debris was pelleted by centrifugation at 20,000xg for 40 min at 4°C. Supernatants were stored at –80°C until further use.

The supernatants containing the GST fusion proteins were applied to GSH Sepharose pre-equilibrated with buffer A at 4°C. After adsorption of the protein, the GSH Sepharose was washed with 100 ml of buffer B (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 150 mM NaCl and 10% (v/v) glycerol, pH 7.5). Bound proteins were eluted in 25 ml of buffer C (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 150 mM NaCl and 10% (v/v) glycerol, pH 8.0) containing 10 mM GSH. The partially purified protein was concentrated to a 1 ml final volume and the protein concentration was determined using the Bradford method (19). Protein was diluted to 0.5 mg/ml and stored at –80°C until further use.

Receptor binding assays

Partially purified GST-hERαdef and GST-rtERdef fusion protein were diluted in TEGD buffer (10 mM Tris pH 7.6, 1.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) containing 1 mg/ml bovine serum albumin as a carrier protein. Receptor binding and competitive binding assays were performed as previously described (8). Briefly, GST-ERdef fusion proteins were diluted in TEGD buffer (10 mM Tris pH 7.6, 1.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) containing 1 mg/ml bovine serum albumin (BSA), and incubated at 37°C for 30 min, 30°C for 1 hr, 20°C for 2 h, and 4°C for 24 h with 0.1-3.5 nM [^{3}H]E2 in 1 ml glass tubes arranged in a 96-well format (Marsh Scientific, Rochester, NY). Fusion protein preparations were diluted to ensure 10,000 dpms of total binding (varied from 1000-2000 fold). Binding assays were initiated by adding 240 µl of

protein preparation to glass tubes containing 5 µl of DMSO and 5 µl [^{3}H]E2, thus the concentration of solvent did not exceed 4 %. Bound [^{3}H]E2 was separated from free using a 96-well filter plate and vacuum pump harvester (Packard Instruments). After drying, the filter plates were sealed and 50 µl of MicroScint 20 scintillation cocktail (Packard Instruments) was added to each well. Bound [^{3}H]E2 was measured using a TopCount luminescense and scintillation counter (Packard Instruments).

Competitive ligand binding assays were performed as described above except diluted GST-ERdef fusion protein preparations were incubated with a final concentration of 2.5 nM [^{3}H]E2 (5 µl aliquot) and increasing final concentrations of unlabeled competitor (0.1 nM – 100 µM, 5 µl aliquots) at 4°C for 24 h. Each treatment was performed in quadruplicate and results are expressed as percent specific binding of [^{3}H]E2 versus log of competitor concentration. IC_{50} values were determined from nonlinear regression for single site competitive binding analysis. The reported IC_{50} values represent the concentration of test compound required to displace 50% [^{3}H]E2 from the GST-ERdef fusion proteins as compared to the 50% displacement of [^{3}H]E2 achieved by unlabeled E2. Analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Cell Culture and transient transfection

Estrogen receptor positive MCF-7 human breast cancer cells (obtained from Dr. L. Murphy, University of Manitoba, at passage 32-35) were maintained with phenol red-free DMEM supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 500 µg/ml gentamicin, 2.5 µg/ml amphotericin B, 100 IU/ml penicillin G, and 100 µg/ml

streptomycin. Cells were cultured in a humidified environment at 37°C in the presence of 5% CO_2 atmosphere.

Transient transfections and gene transcription assays were performed essentially as previously described (20,21). Briefly, MCF-7 cells were seeded at approximately 50% confluency in 6-well tissue culture plates in medium supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and allowed to settle for 7 h. For the preparation of whole cell extracts used in western blot analysis, MCF-7 cells were transiently transfected by the calcium phosphate precipitation method (22) with 5 μg of Gal4-ER expression vector and 0.05 μg of pCMV-lacZ, while for the reporter gene assay, cells were transiently transfected with 1.5 μg of 17m5-G-Luc (provided by Dr. P Chambon), and 0.2 μg of Gal4-hERαdef or 0.2 μg Gal4-rtERdef along with 0.05 μg of pCMV-lacZ. Cells were washed 16 h later with sterile phosphate buffered saline and fresh medium was added to each well. Cells were then treated with E2, or test compound and allowed to incubate for 24 h. Following incubation cells were harvested and assayed for luciferase activity as described previously (23). Each treatment was done in duplicate and two samples were taken from each replicate and each experiment was repeated three times. The concentration required to induce half maximal luciferase activity (EC_{50} value) was calculated using GraphPad 3.0.

Western Blotting

Whole cell extracts from transfected MCF-7 cells were fractionated on a 10% polyacrylamide/SDS gel and transferred to nitrocellulose (Amersham/Pharmacia, Piscataway, NJ). Blots were incubated with anti-Gal4 DNA binding antibody (Santa

Cruz, Santa Cruz, CA) for 2 h at room temperature in the presence of 2% fat-free milk powder and then incubated for 2 h with the secondary conjugated goat anti-(mouse IgG)-horseradish peroxidase (Santa Cruz). Proteins were visualized using ECL detection system (Amersham/Pharmacia) and subsequent exposure to film.

Statistical Analysis

Comparisons of EC_{50} and IC_{50} values between wild type and mutant proteins (both Gal4 ER chimeras and GST fusion proteins) were performed with a one-way ANOVA using the GLM procedure of SAS version 7 (SAS Inc, Cary, NC). Multiple comparisons between wild type (control) and mutant forms of the ER within species were adjusted by Dunnett's method. Comparisons between species were adjusted by the Tukey method. The level of significance was 5 %.

Results

Comparison of the hormone binding properties of GST-hERαdef and GST-rtERdef wild-type and mutant receptors at different temperatures

Sequence alignment identified amino acid residues that line the ligand binding pockets of hERα and rtER and differ between the two receptors i.e. L349 and M528 in hERα and the corresponding residues, M317 and I496 in rtER (Figure 1). Reciprocal mutants were prepared which effectively exchanged the ligand binding pockets of hERα and rtER.

GST-ERdef fusion proteins used in this study exhibit similar purity as previously described (7,8). Mutagenesis did not affect the level of protein expression (data not shown). GST-ERdef fusion proteins were incubated with increasing concentrations of [^{3}H]E2 and the binding affinity was measured *in vitro* at 4, 20, 30, and 37°C using the method of Scatchard (24) (Figure 2 and Table 1). The K_d value of GST-hERαdef wild-type (WT) for E2 was similar to published values (25-27) and was unaffected by temperature. Similarly, the K_d values of the GST-hERαdef mutants differed less than 2-fold when compared to GST-hERαdef WT at the different temperatures examined. However, the affinities of the GST-hERαdef L349M:M528I double mutant and the GST-hERαdef M528I single mutant were significantly lower than GST-hERαdef WT at 4°C.

The K_d value of GST-rtERdef WT was 2-fold lower compared to GST-hERαdef WT at 4°C (Table 1), but comparable to published values (12,28). All three GST-rtERdef mutants displayed significantly higher affinity for

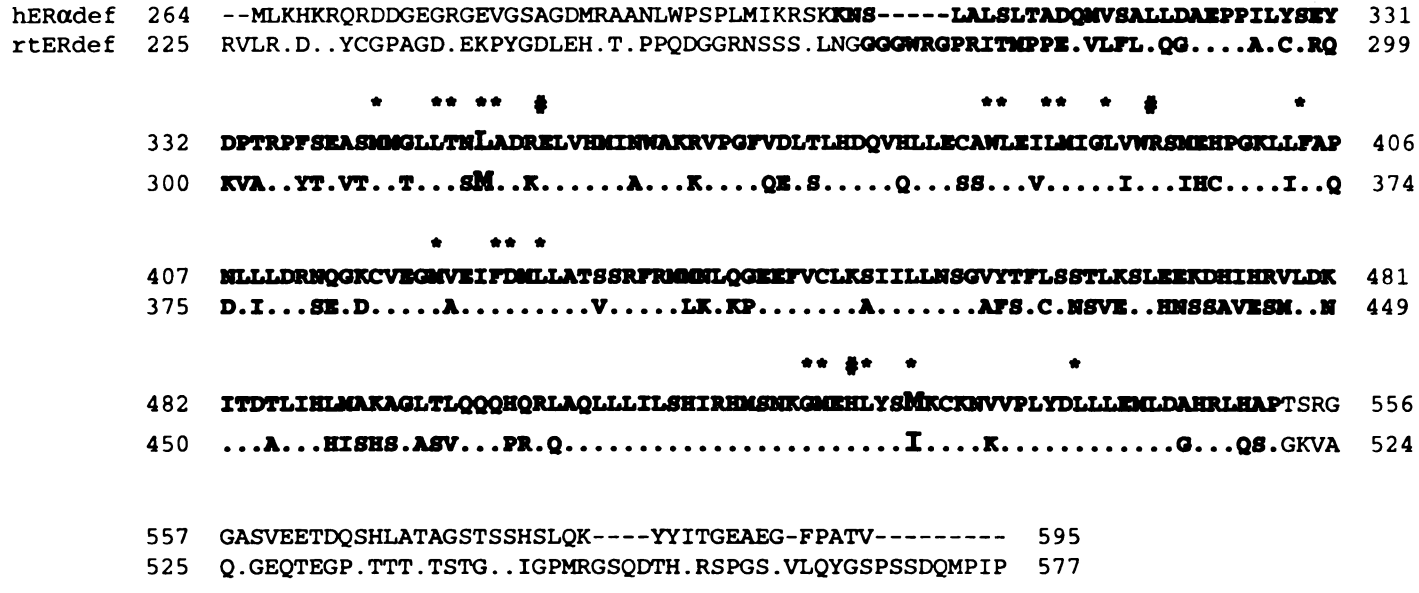

Figure 1. Alignment of the estrogen receptor D, E and F domains from human (hERαdef; GenBank accession no. M11457) and rainbow trout (rtERdef; GenBank accession no. AJ242741). Numbers refer to amino acid position in the full-length sequence. Identical amino acid residues are represented as dots while missing residues are shown as dashes. The E domains are highlighted in bold. Residues involved in hydrogen bonding with 17β-estradiol are identified with a *, while residues, which make hydrophobic contacts are identified with a #. The larger font identifies the amino acid residues that were reciprocally mutated.

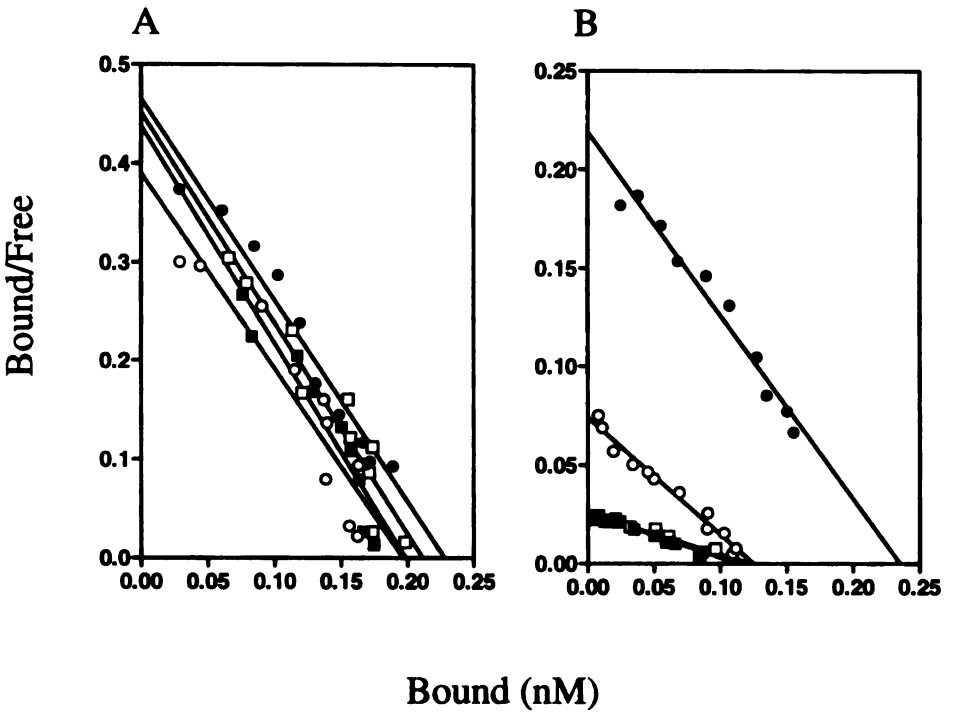

Figure 2. The affinity of wild-type and mutant receptors for 17β-estradiol at different temperatures. Partially purified (A) GST-hERαdef wild-type or (B) GST-rtERdef wild-type were incubated with increasing concentrations of [^{3}H]17β-estradiol at 4°C for 24 h (●), 20°C for 2 h (○), 30°C for 1 h (■) or 37°C for 30 min (□). Affinities of the receptors for E2 were determined by the method of Scatchard {Scatchard, 1949 #209}.

Table 1. Hormone binding of wild-type and mutant ERs at different temperatures. GST-ERdef fusion proteins were incubated with increasing concentrations of [^{3}H]17β-estradiol at 4, 20, 30, or 37°C for 24 h, 2 h, 1 h, or 30 min, respectively. Affinities are expressed as equilibrium dissociation constants (K_d), and represent the mean and standard deviation of three determinations.

Protein Preparation	Average K_d ± S.D. (nM) 4°C	20°C	30°C	37°C
GST-hERαdef				
wild-type	0.50 ± 0.15	0.50 ± 0.10	0.74 ± 0.12	0.57 ± 0.02
L349M	0.52 ± 0.04	0.49 ± 0.07	0.60 ± 0.10	0.64 ± 0.09
M528I	0.92 ± 0.08[a]	0.49 ± 0.13	0.66 ± 0.04[b]	0.76 ± 0.07[b]
L349M:M528I	0.91 ± 0.26[a]	0.92 ± 0.15[a]	0.98 ± 0.05[a]	0.90.± 0.20[a]
GST-rtERdef				
wild-type	1.03 ± 0.14	1.82 ± 0.21	4.58 ± 0.64[d]	5.37 ± 1.25[d]
M317L	0.56 ± 0.04[c]	1.39 ± 0.63	2.27 ± 0.20[c,d]	3.31 ± 0.69[c,d]
I496M	0.82 ± 0.11[c]	1.00 ± 0.05[c]	1.47 ± 0.13[c,d]	1.60 ± 0.11[c,d]
M317L:I496M	0.76 ± 0.08[c]	0.87 ± 0.18[c]	1.53 ± 0.01[c,d]	1.82 ± 0.03[c,d]

[a] $P < 0.05$ vs GST-hERαdef wild-type within each temperature
[b] $P < 0.05$ vs corresponding GST-hERαdef wild-type or mutant protein at 4°C
[c] $P < 0.05$ vs GST-rtERdef wild-type within each temperature
[d] $P < 0.05$ vs corresponding GST-rtERdef wild-type or mutant protein at 4°C

All data analyses were performed using SAS version 7 (SAS Inc, Cary, NC). K_d values were analyzed by a one-way analysis of variance (ANOVA), using the GLM procedure of SAS, with either mutation or temperature as a fixed effect. Pair-wise comparisons between groups and the control (wild-type protein or 4°C) were performed using the Dunnett's test. The level of significance was $P < 0.05$.

E2 than GST-rtERdef WT. The K_d values of GST-rtERdef WT and mutant receptors significantly increased with temperature (Table 1). For GST-rtERdef WT, the K_d value was 5.3-fold higher at 37°C compared to 4°C. The K_d values increased 3.4-fold with the I496M mutation, 1.6-fold with the M317L mutant and less than 1.8-fold for the GST-rtERdef M317L:I496M double mutant at 37°C compared to WT at 4°C. The number of binding sites in GST-hERαdef WT remained constant among the different temperatures (Figure 2). In contrast, GST-rtER WT exhibited dramatic reductions in the number of binding sites at elevated temperatures (Figure 2). A reduction in the number of binding sites at elevated temperature was also seen with the rtER mutants, while only slight reductions were observed with the hERα mutants (data not shown).

Gal4-hERαdef and Gal4-rtERdef wild-type and mutant receptor mediated reporter gene activity at different temperatures

WT and mutant Gal4-ERdef mediated luciferase reporter gene induction was examined at 20, 30, and 37°C for 24 h following treatment with E2. The morphology of the cells was similar at the various temperatures examined. At 37°C cells transfected with Gal4-rtERdef required a 280-fold greater concentration of E2 to achieve half-maximal response compared to Gal4-hERαdef at the same temperature (Figure 3). This difference in transactivation was reduced to 33-fold and to less than 10-fold when transfected MCF-7 cells were maintained at 30 and 20°C, respectively. This was caused by a shift in the dose-response curve for the rtER, since the E2-induced half-maximal response for hERα changed less than 2.5-fold among the three different temperatures examined.

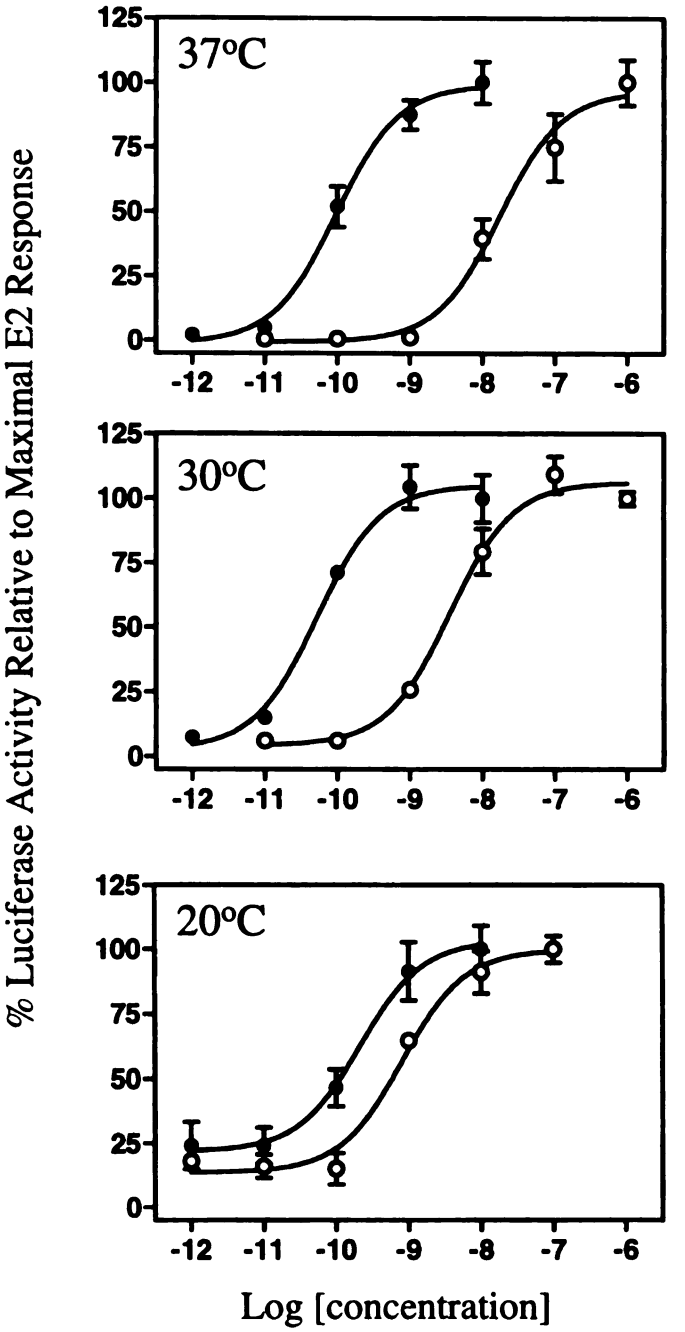

Figure 3. Temperature effects on wild-type human and rainbow trout estrogen receptor-mediated induction of reporter gene activity in the presence of 17β-estradiol. MCF-7 cells were cotransfected with the reporter plasmid 17m5-G-Luc and the Gal4-ERdef expression vector for hERα (●) or rtER (○) for 16 h at 37°C. Following the incubation, cells were rinsed with phosphate buffered saline, fresh media was added and cells were incubated at 20, 30 or 37°C for 24 h in the presence of increasing concentrations of 17β-estradiol. The results shown are from a representative experiment that was repeated two times.

The maximal E2 induction ranged from 20- to 30-fold for Gal4-hERαdef and 50- to 100-fold for Gal4-rtERdef at both 30 and 37°C, but decreased to 4- to 7-fold at 20°C for both receptors. Background luciferase activity for both receptors increased at 20°C, and therefore the data are reported as percent luciferase activity relative to the maximal activity achieved by E2 with each receptor, which was set to 100% for comparative purposes.

No changes in induction of luciferase activity were evident with the Gal4-hERαdef WT and single mutations at 20, 30 or 37°C (Figure 4a). In contrast, the EC_{50} value of the Gal4-hERαdef L349M:M528I double mutant was significantly ($p<0.0001$) increased 20-fold at 37°C (Figure 4a and 5a), but was only slightly increased at 20 and 30°C, indicating that induction of reporter gene activity by the double mutant was dramatically affected by temperature. At 37°C, Gal4-rtERdef M317L:I496M-mediated reporter gene expression increased 5-fold compared to Gal4-rtERdef WT and was only 60-fold lower than that of Gal4-hERαdef WT (Figure 4b and 5b). The difference in transactivation of Gal4-rtERdef M317L:I496M compared to Gal4-hERαdef WT was reduced to 8- and 3-fold at 30 and 20°C, respectively. Interestingly, the transactivation profiles of Gal4-rtERdef M317L:I496M at 20°C and Gal4-hERαdef WT at 37°C were superimposable (Figure 5b), and their respective EC_{50} values were not significantly different ($p>0.05$). Both Gal4-rtERdef M317L and I496M mutations contributed to the decreased transcriptional activity, with the M317L mutation having a greater influence than the I496M mutation (Figure 4b). This is in contrast to the effect on GST-rtERdef binding where the I496M mutation had a greater affinity for E2 compared to the M317L mutant at elevated temperatures (Table 1).

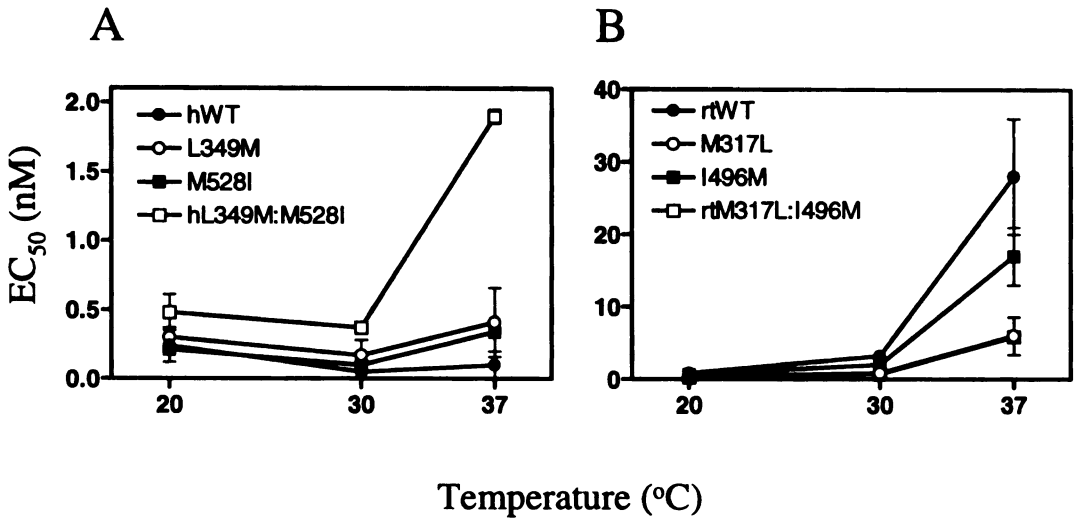

Figure 4. Temperature effects on wild-type and mutant estrogen receptor-mediated 17β-estradiol induced reporter gene activity on EC_{50} values. MCF-7 cells were cotransfected with (A) Gal4-hERαdef wild type (●), L349M (○), M528I (■), or L349M:M528I (□) or (B) Gal4-rtERdef wild type (●), M317L (○), I496M (■), or M317:I496M (□). Following transfection and dosing, cells were incubated for 24 h at 37°C, 30°C or 20°C. Cells were harvested and luciferase and β-galactosidase activity were determined. Though a complete reverse of phenotypes was not seen, the Gal4-hERαdef L349M:M528I double mutant adopted a temperature sensitive phenotype, similar to Gal4-rtERdef WT. Also Gal4-rtERdef M317L:I496M double mutant displayed significantly reduced temperature sensitivity, which was primarily due to the M317L mutation. The results are from a representative experiment that was repeated 3 times.

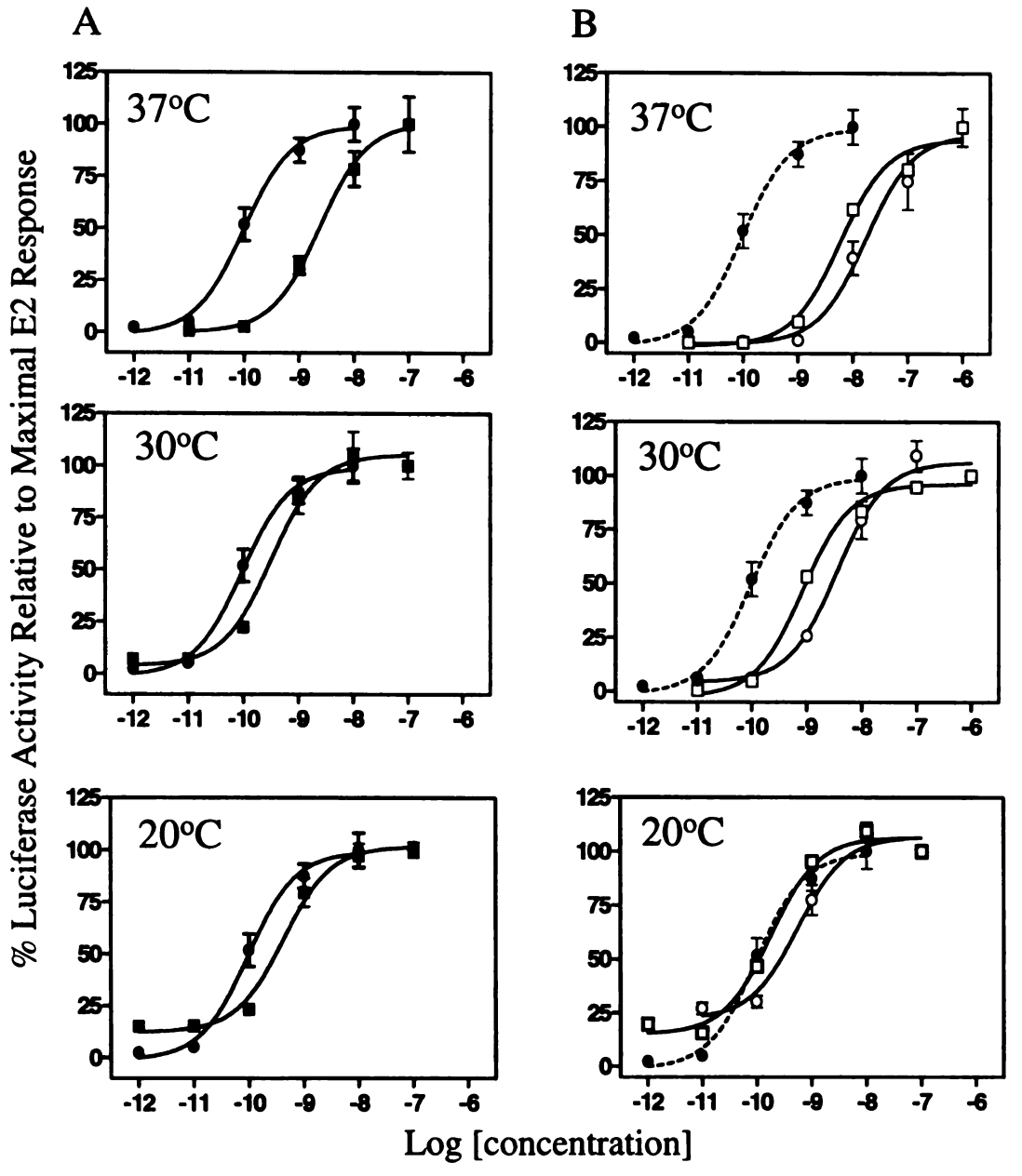

Figure 5. Temperature effects on wild-type and mutant estrogen receptor-mediated 17β-estradiol induced reporter gene activity. MCF-7 cells were cotransfected with Gal4-hERαdef wild-type (●), or Gal4-hERαdef L349M:M528I (■) in panel A, and Gal4-rtERdef wild-type (○) or Gal4-rtERdef M317L:I496M (□) in panel B, as described in Experimental Procedures. The Gal4-hERαdef (●) mediated-reporter gene activity at 37°C (dashed line) in panel B illustrates the improved transactivation ability of wild-type and mutant rtER at the three different temperatures. The results are from a representative experiment that was repeated two times.

The Gal4-rtERdef M317L:I496M double mutant also displayed an increased transcriptional activity of approximately 4-fold compared to Gal4-rtERdef WT at each of the temperatures examined (Figure 4b and 5b). In contrast, the Gal4-hERαdef L349M:M528I displayed a decreased transcriptional activity at the three different temperatures (Figure 4a and Figure 5a), as would be expected from the reciprocal mutagenesis. These data suggest that residues L349/M317 and M528/I496 play important roles in the observed differences in transactivation between hERα and rtER, as well as in influencing the decreased transcriptional activity of the rtER at different temperatures.

Western blot analysis

Western blot analysis was performed to assess the expression level of the WT and mutant Gal4-ERdef proteins. At the three temperatures examined, both Gal4-hERαdef and Gal4-rtERdef WT and mutant proteins were expressed at comparable levels in MCF-7 cells (Figure 6). Therefore, the differences in transactivation observed between Gal4-rtERdef WT and Gal4-hERαdef WT were not due to differences in protein expression levels. However, expression levels for both constructs were always lower at 20°C than at 30 or 37°C, which was consistent with the decreased induction of luciferase activity.

Interaction of estrogenic chemicals with wild-type and mutant receptors

Previous competitive binding studies have shown that α-zearalenol, coumestrol, and 4-*t*-octylphenol exhibit different relative binding affinities for GST-hERαdef WT compared to GST-rtERdef WT (8). Therefore, the ability of these

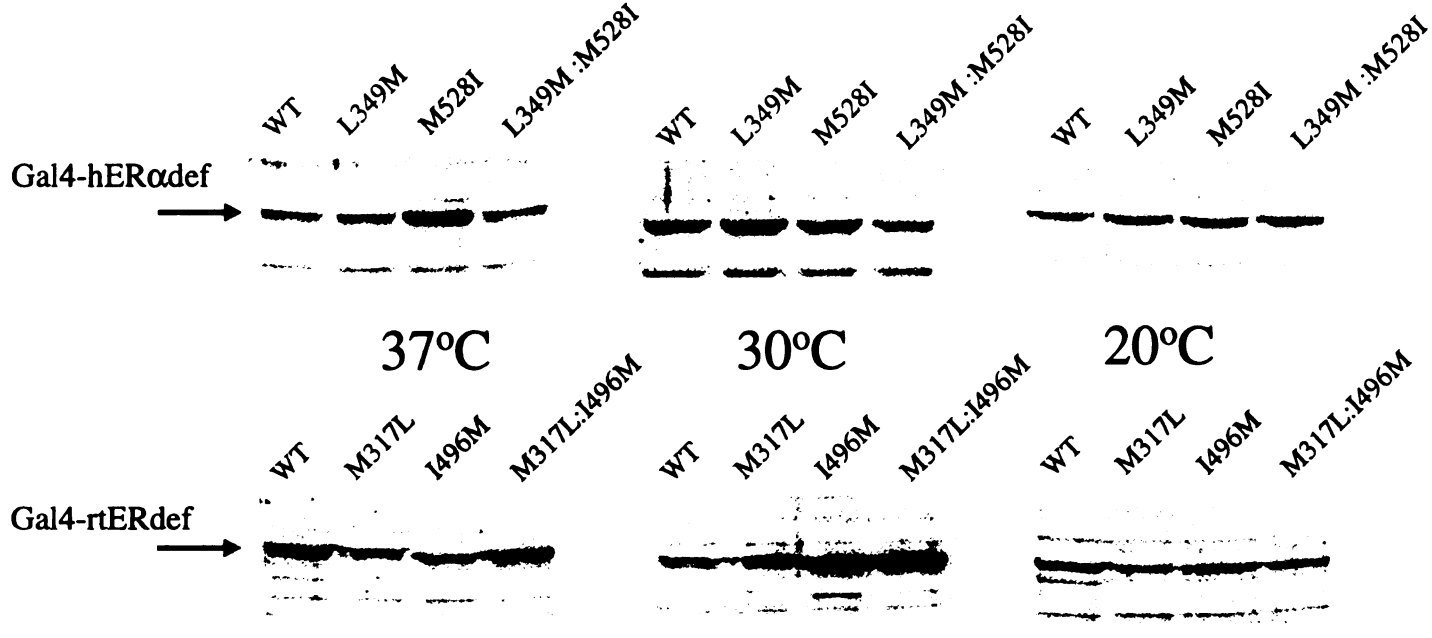

Figure 6. Western blot analysis of wild-type and mutant Gal4-ER expression level. Whole cell extracts were prepared from MCF-7 cells transfected with 5 μg of ER expression plasmid. Extracts were prepared and separated by SDS-PAGE as described in Experimental Procedures. Equal amounts of total protein (10 μg) were loaded per lane, however an exposure five times longer was required for cells incubated at 20°C. The 55 kDa Gal4-hERαdef and 57 kDa Gal4-rtERdef proteins (denoted by arrows) were detected with anti-Gal4 antibody. The results are from a representative experiment that was repeated once.

compounds to compete with [^{3}H]E2 for binding to the mutant receptors was examined in order to investigate the effect of the mutagenesis on the differential ligand binding behaviors of hERα and rtER. Although reciprocal mutagenesis had little effect on the relative binding affinity of α-zearalenol and 4-*t*-octylphenol (data not shown), GST-hERαdef and GST-rtERdef double mutants experienced a nearly complete exchange of binding phenotypes for coumestrol (Table 2 and Figure 7). The IC_{50} value for coumestrol was significantly ($p<0.0001$) lower (10-fold) for GST-hERαdef WT than for GST-rtERdef WT, while the GST-hERαdef WT was not significantly different ($p=0.1554$) from GST-rtERdef M317L:I496M. Similarly, the IC_{50} value for coumestrol was significantly ($p<0.0003$) increased (10-fold) for the GST-hERαdef L349M:M528I double mutant compared to that of GST-hERαdef WT, but was not significantly different compared to that of GST-rtERdef WT.

The ability of coumestrol to induce reporter gene expression mediated by Gal4-ERdef WT and mutant receptors was investigated in order to assess whether the effects observed in competitive binding studies correlated with transactivation activities (Table 2 and Figure 8). Transcriptional activity was examined at the optimal temperatures for Gal4-hERα (37°C) and Gal4-rtERdef (20°C). Similar to the competitive binding data, coumestrol-induced transcriptional activity significantly decreased for the Gal4-hERαdef L349M:M528I double mutant. An increase for the Gal4-rtERdef M317L:I496M double mutant was also observed (Table 2), but was not statistically significant ($p=0.079$). When the transactivation activity of coumestrol mediated by Gal4-rtERdef WT and mutant receptors was examined at 37°C, the same trends seen at 20°C were observed, except that

there was approximately a 10-fold reduction in EC_{50} values for both WT and mutant proteins.

Table 2. Competitive binding and transactivation abilities of wild-type and mutant receptors in response to estrogenic chemicals. The IC_{50} and EC_{50} values represent the mean and standard deviation of 3 and 2 independent experiments, respectively.

	E2		Coumestrol	
Protein Preparation	**IC_{50} (M)**	**EC_{50} (M)**	**IC_{50} (M)**	**EC_{50} (M)**
GST-hERα				
wild-type	$2.2 \pm 0.4 \times 10^{-9}$	$9.8 \pm 9.2 \times 10^{-11}$	$1.2 \pm 0.1 \times 10^{-7}$	$6.1 \pm 3.8 \times 10^{-7}$
L349M	$2.4 \pm 0.2 \times 10^{-9}$	$4.1 \pm 2.5 \times 10^{-10}$	$7.9 \pm 1.5 \times 10^{-7}$[a]	$6.9 \pm 3.8 \times 10^{-7}$
M528I	$2.3 \pm 0.1 \times 10^{-9}$	$3.4 \pm 0.3 \times 10^{-10}$	$3.0 \pm 0.7 \times 10^{-7}$	$7.1 \pm 3.4 \times 10^{-7}$
L349M:M528I	$3.4 \pm 1.0 \times 10^{-9}$[a]	$1.9 \pm 0.5 \times 10^{-9}$[b]	$1.2 \pm 0.3 \times 10^{-6}$[a]	$2.9 \pm 0.5 \times 10^{-6}$[b]
GST-rtER				
wild-type	$2.7 \pm 0.2 \times 10^{-9}$	$1.0 \pm 0.4 \times 10^{-9}$	$1.1 \pm 0.1 \times 10^{-6}$	$5.1 \pm 2.1 \times 10^{-7}$
M317L	$2.5 \pm 0.1 \times 10^{-9}$	$2.8 \pm 0.4 \times 10^{-10}$[d]	$8.7 \pm 1.1 \times 10^{-7}$[c]	$8.8 \pm 1.6 \times 10^{-8}$
I496M	$2.8 \pm 0.5 \times 10^{-9}$	$5.6 \pm 0.2 \times 10^{-10}$	$4.0 \pm 1.5 \times 10^{-7}$[c]	$1.4 \pm 0.3 \times 10^{-7}$
M317L:I496M	$3.2 \pm 0.6 \times 10^{-9}$	$3.6 \pm 0.8 \times 10^{-10}$[d]	$4.6 \pm 1.5 \times 10^{-7}$[c]	$7.2 \pm 2.2 \times 10^{-8}$

[a] $P < 0.05$ vs GST-hERαdef wild-type [b] $P < 0.05$ vs Gal4-hERαdef wild-type
[c] $P < 0.05$ vs GST-rtERdef wild-type [d] $P < 0.05$ vs cGal4-rtERdef wild-type

All data analyses were performed using SAS version 7 (SAS Inc, Cary, NC). IC_{50} or EC_{50} values were analyzed by a one-way analysis of variance (ANOVA), using the GLM procedure of SAS, with either mutation or temperature as a fixed effect. Pair-wise comparisons between groups and the control (wild-type protein) were performed using the Dunnett's test. The level of significance was $P < 0.05$.

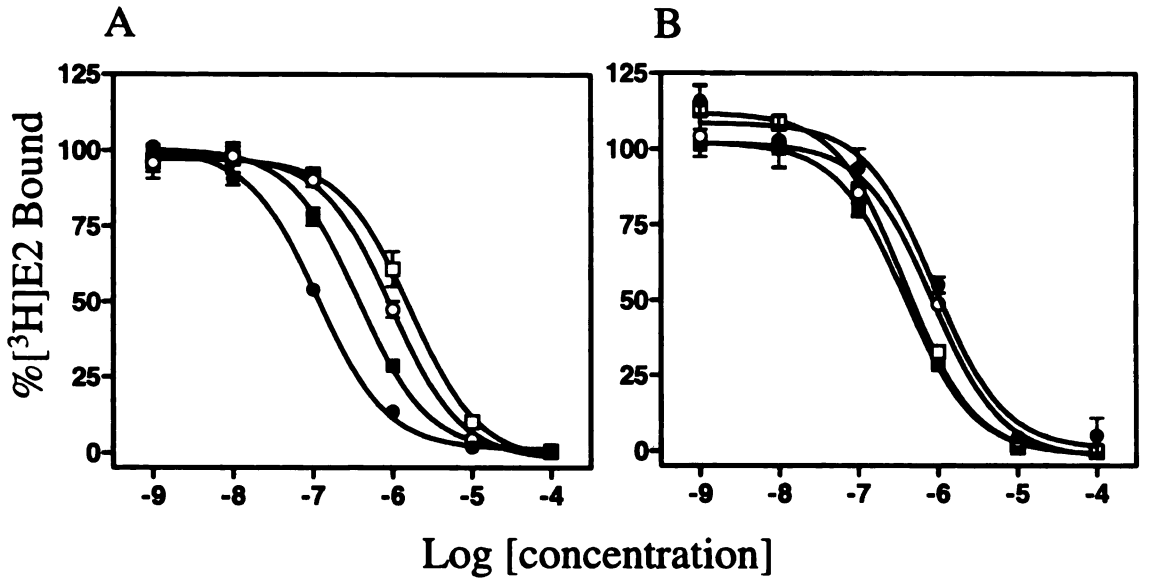

Figure 7. Effect of reciprocal mutagenesis on the competitive binding ability of coumestrol. Representative competitive binding curves of coumestrol with (A) GST-hERαdef wild type (●), L349M (○), M528I (■), and L349M:M528I (□), or (B) GST-rtERdef wild type (●), M317L (○), I496M (■), and M317:I496M (□). The results are from a representative experiment that was repeated at least two times.

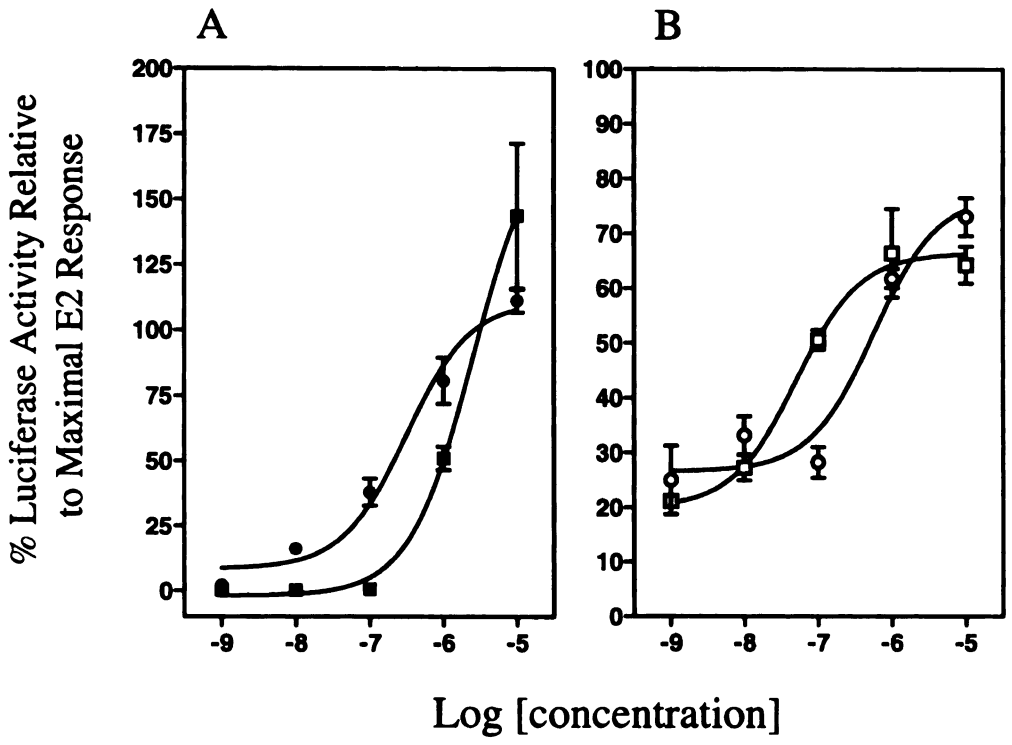

Figure 8. Transactivation abilities of wild-type and mutant receptors in response to coumestrol. MCF-7 cells were cotransfected with (A) Gal4-hERαdef wild-type (●) Gal4-hERαdef L349M:M528I (■), or (B) Gal4-rtERdef wild-type (○) Gal4-rtERdef M317I:I496M (□), as described in the Materials and Methods. Following transfection and dosing, cells were transfected with Gal4-hERαdef were incubated for 24 h at 37°C, while cells transfected with rtERdef were incubated for 24 h at 20°C. The results are from a representative experiment that was repeated two times.

Discussion

In order to focus on differences in ligand binding and transactivation ability between hERα and rtER that may reside within their respective ligand binding domains, GST-ER and Gal4-ER fusion proteins consisting of the D, E, and F domains of each receptor were constructed. This ensured that effects imposed by other domains, such as the DNA binding domain would not influence the results (16). The GST-ERdef and Gal4-ERdef fusion proteins used in this study exhibited similar E2 affinity and transactivation ability when compared to full-length ERs (25-27).

Saturation analysis confirmed that GST-rtERdef exhibited reduced binding affinity for E2 at elevated temperatures. Gal4-rtERdef transactivation ability was also significantly compromised, as greater E2 concentrations were required to induce a half-maximal response in transfected cells maintained at 37°C compared to 20°C. The results demonstrate that the rtERdef exhibits reduced E2 binding affinity and transactivation ability at elevated temperatures, both of which reflect the loss of protein secondary structure. In contrast, the binding affinity and the transactivation ability of hERαdef were not affected by temperature, indicating that the hERαdef exhibits greater stability across a wider range of temperatures. .

The enhanced activity of rtER at lower temperatures has been previously observed in transfected fish and human cell lines (9) and, to a lesser extent, in yeast grown at 30°C (10). These results are not surprising since the optimal temperature for maintaining rainbow trout is 16 ±1 °C. Similarly, the transactivation ability of another piscine ER, the Oreochromis aureus (OaER), also exhibits reduced activity at temperatures above its normal physiological range (29). Moreover, other fish proteins

have also been shown to function more efficiently at lower temperatures compared to their mammalian counterparts (13-15). For example, incubation temperature was shown to influence cytochrome P450 activity in rainbow trout hepatocytes, with lower temperatures producing greater activity (13). Rapid inactivation of dogfish shark P450 enzyme activity in liver microsomes was observed at 38°C, while in rabbit microsomes rapid inactivation occurs at 48°C and the reaction is still linear at 43°C (15). Rainbow trout ATPase activity has also been shown to be more heat-sensitive than that of rabbit ATPase (14). Although a substantial amount of experimental evidence has been accumulated on protein thermal stability (sequence, structure, mutagenesis, and thermodynamics), no single mechanism or motif has been identified that is responsible for protein thermal stabilization (30). Thermal stabilization is a combination of intrinsic (hydrogen bonds, salt bridges, and hydrophobic effects) and extrinsic characteristics (31).

The hERα and rtER E domains share 60% sequence identity with only two residue differences, L349 and M528 in hERα and M317 and I496 in rtER, in their respective ligand binding pockets (8). Results obtained with the rtERdef reciprocal mutants demonstrate that M317 and I496 affect ligand binding and transactivation ability at different temperatures. In general, the introduction of human residues, M317L and I496M, resulted in the rtERdef mutant displaying the phenotype of hERαdef. The rtER M317L:I496M double mutant exhibited increased E2 affinity and transactivation compared to rtERdef WT at all temperatures examined. In addition, increasing the incubation temperature had a less pronounced effect on reducing the E2 affinity of the rtERdef M317L:I496M double mutant. This increase in thermal stability may be a result of ligand-stabilized protein secondary structure since the rtERdef M317L:I496M double

mutant displayed a significantly higher affinity for E2 compared to WT. The hERαdef L349M:M528I double mutant showed modest rtER phenotypic behavior and also exhibited significantly decreased E2 binding affinity and E2-induced transactivation ability at 37°C. Decreasing temperatures increased the transactivation ability of the hERαdef L349M:M528I double mutant, as was seen with rtERdef WT.

Interestingly, exchanging a 35 amino acid stretch, (β3 region) between the OaER and the chicken ER (cER) partially rescued the thermal deficient transactivation of the OaER (29), confirming that reciprocal mutagenesis of residues L349, M528 in hERα and M317, I496 in rtER is important but not sufficient for phenotypic reciprocity. The β3 region, which encompasses amino acid residues Glu444 to Val478 and corresponds to helix 9 and half of helix 10 of hERα, is highly conserved among the different ERαs, with only two amino acid residue differences between hERα and cER. However, only 46% of the residues are conserved in the OaER β3 region. Similarly, the rtER β3 region also shares low identity (49%) relative to hERα and cER, compared to 69% identity with the OaER β3 region (29). Although hERα and hERβ isoforms only share 31% identity within the β3 region, there have been no reports of hERβ displaying any temperature deficient phenotypes. This suggests that variability within the β3 region may not be sufficient for temperature sensitivity, though in certain contexts it can contribute to thermal stabilization (29). The OaER also contains Met304, analogous to Met317 in rtER, suggesting that it may also contribute to thermal instability. It is interesting to speculate that the combination of reciprocal mutagenesis with β3 region exchange as described by Tan and coworkers (29), could lead to complete reciprocity of the temperature deficient transactivation phenotype.

Two amino acid substitutions also exist within the hERα and hERβ binding pockets, Leu384 and Met421 in ERα are replaced by Met336 and Ile373 in ERβ (17). Both receptors also share a similar degree (i.e. 58%) of sequence identity in their E domains (32) compared to hERα and rtER. The residues used in the reciprocal mutants for the present study and the residue substitutions in the ERα and ERβ binding pockets are considered to be conservative, but will affect the volume and hydrophobic character of the binding pocket (17,33). Moreover, the greater relative binding affinity of genistein for ERβ compared to ERα has been attributed to these two residue differences (17,34).

Although phenotype exchange was most evident in the double mutants, examination of single mutant data suggests that rtER I496M and hERα M528I made greater contributions to the adopted binding phenotype while rtER M317L and hERα L349M appeared to contribute more to the adopted transactivation abilities. This is consistent with the location of residue M528 in hERα, which occurs in a region (amino acids 515-535) that is important in the recognition of structurally diverse estrogens and antiestrogens (35). Existing crystal structures for the hERα E domain bound with E2 reveal that M528 interacts with the D-ring while L349 makes contact near the A-ring of the steroid (36,37). Other non-conservative mutations (i.e. C447A, K449E, M528A) in this region also resulted in ER phenotypes that exhibited compromised transactivation ability (38) and instability at elevated temperatures (27,39,40). Since the effect of the reciprocal mutagenesis was significantly more pronounced in rtER compared to hERα, factors outside of the binding pocket also influence E2 binding affinity and transactivation ability at different temperatures.

Mutations in hERαdef that affected the relative binding affinity of coumestrol, also resulted in a corresponding change in its transactivation ability (Table 2). However, for selected ligands, the large volume of the ER binding pocket appears to allow the mutations to exert differential effects that alter transactivation ability without corresponding changes to binding affinity. For example, a mutation of M532 in mouse ERα, analogous to M528 in hERα, diminished the transactivation response of diethylstilbestrol 7.5-fold but enhanced indenestrol A response 40-fold, with negligible changes in binding affinity (41).

Single amino acid differences within nuclear receptors from different species have been previously identified (42). Reciprocal mutagenesis in the human and chicken progesterone receptor has been shown to affect sensitivity to the abortifacient, RU486 (43). The human and chicken peroxisome proliferator-activated receptors also undergo a reversal of phenotypes following reciprocal mutagenesis of a single amino acid residue (44). Although reciprocal mutagenesis of the rtER and hERα in this study did not result in a complete reciprocity of phenotypes, significant alterations in behavior were observed. In the absence of structural data, the key ligand-protein interactions and the consequences of these mutations on protein structure, as well as subsequent ligand binding and transactivation ability at different temperatures, will be difficult to ascertain. Nevertheless, results from this study demonstrate that L349 and M528, and M317 and I496 play important roles in the differences observed between hERα and rtER, respectively, at different temperatures. Other binding pocket residue differences among species may reveal additional ligand-protein interactions that may provide further insights into species-specific ligand-induced receptor phenotypes.

This report identifies two amino acid residues that contribute not only to differences in binding and transactivation between hERα and rtER but also play significant roles in the reduced activity of the rtER at elevated temperatures. The biological relevance of the rtER exhibiting reduced function at elevated temperature is unclear, but may involve increased flexibility in the binding pocket of the rtER compared to hERα, that causes the rtER to become unstable at elevated temperatures. The present report also highlights the impact of temperature when comparing functional characteristics of proteins from poikilothermic species, such as rainbow trout, and humans.

References

1. Evans, R. (1988) *Science* **240,** 889-895.

2. Carson-Jurica, M. A., Schrader, W.T., O'Malley, B.W. (1990) *Endocr Rev* **11**(2), 201-220.

3. Beato, M. (1991) *FASEB J* **5**(7), 2044-2051.

4. Tenbaum, S., and Baniahmad, A. (1997) *Int J Biochem Cell Biol* **29**(12), 1325-1341.

5. Mosselman, S., Polman, J., and Dijkema, R. (1996) *FEBS Lett* **392**(1), 49-53.

6. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J.A. (1996) *Proc Natl Acad Sci U S A* **93**(12), 5925-5930.

7. Matthews, J. B., and Zacharewski, T. R. (2000) *Toxicol Sci* **53**(2), 326-339.

8. Matthews, J. B., Celius, T., Halgren, R., and Zacharewski, T. R. (2000) *J Steroid Biochem Mol Bio* **74**(4), 223-234.

9. Le Drean, Y., Kern, L., Pakdel, F., and Valotaire, Y. (1995) *Mol. Cell. Endocrinol.* **109,** 27-35.

10. Petit, F., Valotaire, Y., and Pakdel, F. (1995) *Eur J Biochem* **233**(2), 584-592.

11. Pakdel, F., Le Guellec, C., Vaillant, C., Le Roux, M., and Valotaire, Y. (1989) *Mol Endocrinol* **3**(1), 44-51.

12. Pakdel, F., Le Gac, F., Le Goff, P., and Valotaire, Y. (1990) *Mol Cell Endocrinol* **71**(3), 195-204.

13. Klaunig, J. E., Ruch, R. J., and Goldblatt, P. J. (1985) *In Vitro Cell Dev Biol* **21**(4), 221-228.

14. de Toledo, F. G., Albuquerque, M. C., Goulart, B. H., and Chini, E. N. (1995) *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **111**(1), 93-98.

15. Pohl, R. J., Bend, J. R., Guarino, A. M., and Fouts, J. R. (1974) *Drug Metab Dispos* **2**(6), 545-555.

16. Petit, F. G., Valotaire, Y., and Pakdel, F. (2000) *Nucleic Acids Res* **28**(14), 2634-42.

17. Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J., and Carlquist, M. (1999) *Embo J* **18**(17), 4608-4618.

18. Gillesby, B., and Zacharewski, T. R. (1999) *Breast Cancer Res Treat* **56**, 253-265.

19. Bradford, M. M. (1976) *Anal Biochem* **72**(2), 248-254.

20. Balaguer, P., Joyeux, A., Denison, M.S., Vincent,R., Gillesby, B.E., Zacharewski, T. (1996) *Can J Physiol Pharmacol* **74**(2), 216-222.

21. Zacharewski, T. R., Meek, M.D., Clemons, J.H., Wu, Z.F., Fielden, M.F., Matthews, J.B. (1998) *Toxicol Sci* **46**, 282-293.

22. Sambrook, J., Fristch, E.F., Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual.*, Cold Spring Harbor Laboratory Press, New York.

23. Brasier, A. R., Tate, J.E., Habener, J.F. (1989) *Biotechniques* **7**(10), 1116-1122
24. Scatchard, G. (1949) *Ann NY Acad Sci* **51**, 660-672.

25. Wooge, C., H,. Nilsson, G,M,. Heierson, A., McDonnell, D.P., Katzenellenbogen, B.S. (1992) *Mol Endocrinol* **6**(6), 861-869.

26. Jaglaguier, S., Mesnier, D., Lager, J. L., and Auzou, G. (1996) *J Steroid Biochem Mol Biol* **57**(1-2), 43-50.

27. Reese, J. C., and Katzenellenbogen, B. S. (1992) *J Biol Chem* **267**(14), 9868-9873.

28. Donohoe, R. M., and Curtis, L. R. (1996) *Aquatic Toxicol* **36**, 31-52.

29. Tan, N. S., Frecer, V., Lam, T. J., and Ding, J. L. (1999) *Biochim Biophys Acta* **1452**(2), 103-120.

30. Kumar, S., Tsai, C. J., and Nussinov, R. (2000) *Protein Eng* **13**(3), 179-191.

31. Vieille, C., Burdette, D. S., and Zeikus, J. G. (1996) *Biotechnol Annu Rev* **2**(3), 1-83.

32. Pace, P., Taylor, J., Suntharalingam, S., Coombes, R. C., and Ali, S. (1997) *J Biol Chem* **272**(41), 25832-25838.

33. Stickle, D. F., Presta, L. G., Dill, K. A., and Rose, G. D. (1992) *J Mol Biol* **226**(4), 1143-1159.

34. Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998) *Endocrinology* **139**(10), 4252-4263.

35. Ekena, K., Weis, K. E., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1997) *J Biol Chem* **272**(8), 5069-5075.

36. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**(6652), 753-758.

37. Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) *Proc Natl Acad Sci U S A* **95**(11), 5998-6003.

38. Ekena, K., Weis, K. E., Katzenellenbogen, J., A.,, and Katzenellenbogen, B. S. (1996) *J Biol Chem* **27**(33), 20053-20059.

39. Reese, J. C., and Katzenellenbogen, B. S. (1991) *J Biol Chem* **266**(17), 10880-10887.

40. Pakdel, F., Reese, J.C., Katzenellenbogen, B.S. (1993) *Mol Endocrinol* **7**(11), 1408-1417.

41. Kohno, H., Bocchinfuso, W. P., Gandini, O., Curtis, S. W., and Korach, K. S. (1996) *J Mol Endocrinol* **16**(3), 277-285.

42. Govindan, M. V. (1990) *Mol Endocrinol* **4**(3), 417-427.

43. Benhamou, B., Garcia, T., Lerouge, T., Vergezac, A., Gofflo, D., Bigogne, C., Chambon, P., and Gronemeyer, H. (1992) *Science* **255**(5041), 206-209.

44. Takada, I., Yu, R. T., Xu, H. E., Lambert, M. H., Montana, V. G., Kliewer, S. A., Evans, R. M., and Umesono, K. (2000) *Mol Endocrinol* **14**(5), 733-740

CHAPTER 7

In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors α and β

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In Vitro and in Vivo Interactions of Bisphenol A and Its Metabolite, Bisphenol A Glucuronide, with Estrogen Receptors α and β

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The estrogenic activities of bisphenol A (BPA) and its major metabolite BPA glucuronide (BPA-G) were assessed in a number of in vitro and in vivo assays. BPA competed with [^{3}H]-17β-estradiol (E2) for binding to mouse uterine cytosol ER, a glutathione *S*-transferase (GST)−human ER D, E, and F domain fusion protein (GST−hERαdef) and full-length recombinant hERβ. The IC_{50} values for E2 were similar for all three receptor preparations, whereas BPA competed more effectively for binding to hERβ (0.96 μM) than to either mouse uterine cytosol ER (26 μM) or GST−hERαdef (36 μM). In contrast, BPA-G did not competitively displace [^{3}H]E2 from any of the ER preparations. In MCF-7 cells transiently transfected with Gal4-hERαdef or Gal4-hERβdef, BPA induced reporter gene activity with comparable EC_{50} values (71 and 39 μM, respectively). No significant induction of reporter gene activity was seen for BPA-G. Cotreatment studies showed that concentrations of (10 μM) BPA and BPA-G did not antagonize E2-induced luciferase mediated through either Gal4-hERαdef or Gal4-hERβdef. In vivo, the uterotropic effect of gavage or subcutaneous (sc) administration of 0.002−800 mg of BPA/kg of body weight/day for three consecutive days was examined in immature rats. Dose-related estrogenic effects on the rat uterus were observed at oral doses of 200 and 800 mg/kg and at sc doses of 10, 100, and 800 mg/kg. These results demonstrate that BPA competes more effectively for binding to ERβ, but induces ERα- and ERβ-mediated gene expression with comparable efficacy. In contrast, BPA-G did not exhibit any in vitro estrogenic activity. In addition, there was a clear route dependency on the ability of BPA to induce estrogenic responses in vivo.

Introduction

Accumulating evidence suggests that exposure to natural and synthetic chemicals that mimic the activity of 17β-estradiol (E2) may adversely affect wildlife and human health. Although controversial (*1*), there have been reports of decreases in sperm production and seminal volume in humans during the past half-century (*2*−*4*). In wildlife, field studies indicate there are increases in reproductive abnormalities in mammals (*5*), reptiles (*6*), birds (*7*), and several fish species (*8*) following exposure to estrogenic environmental contaminants. Many of these effects are thought to occur through an estrogen receptor (ER)-mediated mechanism of action.

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The ER is a member of the nuclear receptor superfamily, a family of nuclear proteins that function as transcription factors to modulate gene expression in a ligand-dependent manner (*9*). The two subtypes, ERα and ERβ, are products of distinct genes and differ in their tissue distribution, and in their ligand preferences (*10*, *11*). For example, in rat, ERα is expressed at higher levels in the uterus, kidney, and epididymis, while ERβ levels are higher in the prostate, ovaries, and lung (*12*). As with other members of the nuclear receptor superfamily, the ER has a modular structure consisting of six domains (A–F) (*9*, *13*). The highly conserved DNA-binding domain (C domain) separates a highly variable NH_2-terminal region (A and B domains), which contains a ligand-independent activation region (AF-1), and a COOH-terminal region, which includes a hinge region (D domain), the ligand binding domain (E domain), and a variable F domain. The ER E domain also harbors a nuclear localization signal, a dimerization region, and a ligand-dependent activation region (AF-2) as well as residues which interact with heat shock protein 90 (hsp90) (*14*–*16*). When estrogen binds, the ER undergoes a conformational change, which allows it to bind to its cognate DNA target site, termed estrogen responsive elements (EREs), located in the regulatory region of estrogen-inducible genes, thereby modulating the expression of estrogen responsive genes.

Bisphenol A [4,4′-isopropylidene-2-diphenol (BPA)], a monomer used in the production of polycarbonate and epoxy resins, has been shown to elicit ER-mediated activity in a number of in vitro and in vivo assays. BPA has been shown to compete with [^{3}H]E2 for binding to ERα and ERβ (*11*), and induce a number of in vitro effects, including ERα- and ERβ-mediated reporter gene activity (*11*), MCF-7 human breast cancer cell proliferation, progesterone receptor expression (*17*), vitellogenin expression in carp hepatocytes (*18*), and prolactin release in a pituitary tumor cell line (*19*).

High doses of BPA have been reported to elicit reproductive toxicity and abnormal cellular development in rodents (*20*). However, unlike E2, BPA had no effect on uterine weight in exposed rats at doses as high as 150 mg/kg of body weight, but induced peroxidase activity and elevated progesterone receptor (PR) levels similar to that of E2 (*21*). Furthermore, in cotreatment studies, BPA antagonized the E2 stimulatory effects on both peroxidase activity and PR levels but did not inhibit E2-induced increases in uterine weight, suggesting that BPA may use a mechanism of action distinct from that of E2 (*21*).

BPA is readily metabolized in vivo through glucuronidation to BPA glucuronide (BPA-G), and subsequently excreted in the feces and urine (*22*, *23*). Pharmacokinetic studies have shown that there is a clear route dependency in the bioavailability of parent BPA, with considerably higher systemic blood concentrations of BPA after sc and intraperitoneal administration than after oral administration (*23*).

There has been increasing concern regarding the estrogenic activities of BPA as a result of reports that trace levels can leach from the lining of food cans, polycarbonate plastic ware, and dental resins (*24*, *25*). Consequently, the estrogenic activities of BPA and BPA-G were investigated for their ability to compete with [^{3}H]E2 for binding to the recombinantly expressed glutathione *S*-tranferase (GST)–estrogen receptor α (ERα) fusion protein, consisting of the D–F domains of hERα linked to GST, and to full-length ERβ proteins, to induce and antagonize ERα- and ERβ-mediated reporter gene expression in MCF-7 cells transfected with Gal4-hERαdef or Gal-hERβdef and the Gal4-regulated luciferase reporter gene (17m5-G-Luc) in vitro. To account for pharmacodynamic and pharmacokinetic interactions that may affect in vivo estrogenic activity, the ability of BPA to induce a variety of uterine responses was investigated in immature Sprague-Dawley rats using two different routes of exposure.

Experimental Procedures

Chemicals. Bisphenol A (99.9% pure, Bayer AG) and bisphenol A glucuronide were provided by the BPA Global Industry Group. Dimethyl sulfoxide (DMSO) and 17β-estradiol (E2) used in the in vitro studies were obtained from Sigma (St. Louis, MO), and E2 used in the in vivo studies was from Sigma (Poole, Dorset, U.K.). [2,4,6,7,16,17-^{3}H]E2 (123 Ci/mmol) was purchased from New England Nuclear (Boston, MA). MicroScint 20 was obtained from Packard Instruments (Meriden, CT). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA), and restriction enzymes were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). Phenol red-free Dulbecco's modified Eagle's medium (D-MEM) and medium supplements were from Life Technologies (Gaithesburg, MD). Fetal bovine serum (FBS) and D-luciferin were purchased from Intergen (Purchase, NY) and Molecular Probes (Eugene, OR), respectively. All other chemicals and biochemicals were of the highest quality available from commercial sources.

Animals. Immature female Alpk:APfSD rats (21–22 days old) with body weights of 38–53 g were obtained from the breeding unit at AstraZeneca (Alderley Park, U.K.). Animals were housed in multiple rat racks suitable for animals of this strain and the weight range expected during the course of the study. Humidity was controlled, and a 12 h light/dark cycle was maintained. Animals were weaned on Rat and Mouse No. 3 (RM3) breeding diet (Special Diets Services Ltd., Witham, Essex, U.K.) until they were transferred to AstraZeneca Central Toxicology Laboratory (CTL) and were then maintained on Rat and Mouse No. 1 (RM1) maintenance diet, as previously recommended (*26*). All animals were acclimatized to the laboratory for at least 18 h before being treated.

Construction of Plasmids. The plasmid pGEX-hERαdef was constructed as previously described (*27*). The plasmid pGal4-hERβdef (amino acids 204–530) was constructed by PCR amplification of the hERβ (kindly provided by L. Murphy, University of Manitoba, Winnipeg, MB), using the primers pr-hf (5′-caaactcgagcctgccgacttcggaagtgttacga-3′) and pr-hr (5′-caaaggatcctcactgagactgtgggttctgg-3′). The fragment was digested with *Xho*I and *Bam*HI and ligated into the similarly digested eukaryotic expression vector containing the DNA binding domain of the yeast transcription factor Gal4, pG4MpolyII (kindy provided by P. Chambon, IGBMC CNRS-LGME, Illkirch Cedex C. U. de Strasbourg, France). PCR amplification was performed essentially as previously described (*28*) using Vent DNA polymerase in a reaction mixture containing Thermopol buffer, 200 μM dNTPs, 1 mM $MgSO_4$, 500 nM primer, and 1.25 units of polymerase, which was heated to 94 °C for 5 min followed by 35 rounds at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 105 s. The sequence of each construct was confirmed with restriction enzyme digestion and ABI/Prism automated sequencing (Perkin-Elmer Applied Biosystems, Foster City, CA).

Expression and Purification of the GST–ER Fusion Protein. Expression, purification, and characterization of the GST–hERαdef fusion protein were carried out as previously described (*27*).

Receptor Binding Assays. The partially purified GST–hERαdef fusion protein or recombinant hERβ (PanVera, Madison, WI) was diluted in TEGD buffer [10 mM Tris (pH 7.6), 1.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] containing 1 mg/mL bovine serum albumin (BSA) as a carrier protein. For

mouse uterine cytosol preparation, immature female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in a controlled environment (40–60% humidity; 20–22 °C ambient temperature) on a 12 h light/dark cycle. Harlen Teklad 22/5 Rodent Diet 8640 (Madison, WI) and tap water were provided ad libitum. Uterine tissue from 21-day-old CD-1 mice was excised, trimmed of excess fat and connective tissue, weighed, and homogenized in 1.0 mL of TEGD [10 mM Tris base, 1.5 mM EDTA, 10% glycerol, and 1.0 mM DTT (pH 7.6)] per 50 mg of uterine tissue with 3 × 20 s bursts using a Brinkman Polytron homogenizer at 50% output. Samples were centrifuged at 3000*g* for 10 min at 4 °C. The supernatant (cytosol) was centrifuged at 105000*g* for 1 h at 4 °C. The protein concentration of the cytosol was adjusted to 2.0 mg/mL and the protein stored at −80 °C.

Protein preparations and the mouse uterine cytosol were incubated at 4 °C for 24 h and at 30 °C for 2 h, respectively, with 2.5 nM [^{3}H]E2 and increasing concentrations of unlabeled competitor in 1 mL glass tubes arranged in a 96-well format (Marsh Scientific, Rochester, NY). Protein preparations were diluted to ensure 10 000 dpm of total binding (dilutions were 500-fold hERβ and 1500-fold GST−hERαdef). Binding assays were initiated by adding 240 μL of the protein preparation to glass tubes containing 5 μL of DMSO and 5 μL of [^{3}H]E2; thus, the solvent concentration did not exceed 4%. The amount of nonspecific binding was determined in the presence of a 400-fold excess of unlabeled E2. Bound [^{3}H]E2 was separated from free [^{3}H]E2 using a 96-well filter plate and vacuum pump harvester (Packard Instruments). Filter plates containing the protein were washed with 3 × 50 mL of TEG [10 mM Tris buffer (pH 7.6), 1.5 mM EDTA, and 10% (v/v) glycerol] and allowed to dry under continued suction for 30 s. After drying, the undersides of the filter plates were sealed and 50 μL of MicroScint 20 scintillation cocktail was added to each well. The amount of bound [^{3}H]E2 was measured using a TopCount luminescense and scintillation counter (Packard Instruments).

Each treatment was performed in quadruplicate, and results are expressed as the percent specific binding of [^{3}H]E2 versus the log of the competitor concentration. The reported IC_{50} values represent the concentration of test compound required to displace 50% of the [^{3}H]E2 from the ER preparation as compared to the 50% displacement of [^{3}H]E2 achieved by unlabeled E2. These analyses were performed using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA).

Cell Culture. MCF-7 human breast cancer estrogen receptor positive cells (obtained from L. Murphy, University of Manitoba, at passage 32–35) were maintained with phenol red-free D-MEM supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 500 μg/mL gentamicin, 2.5 μg/mL amphotericin B, 100 IU/mL penicillin G, and 100 μg/mL streptomycin. Cells were cultured in a humidified environment at 37 °C with 5% CO_2.

Transfection and Reporter Gene Assays. Transient transfections and gene transcription assays were performed essentially as previously described (*29*, *30*). Briefly, MCF-7 cells were seeded at approximately 50% confluency in six-well tissue culture plates in medium supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and allowed to settle for 7 h. Cells were transiently transfected by the calcium phosphate coprecipitation method (*31*) with 2.5 μg of 17m5-G-Luc, 0.2 μg of Gal4-hERαdef, or 0.2 μg Gal4-hERβdef, along with 0.01 μg of pCMV-lacZ (β-galactosidase expression vector). Cells were washed 16 h later with sterile phosphate-buffered saline (PBS), and then fresh medium was added to each well.

Transiently transfected MCF-7 cells were exposed to final concentrations ranging (i) from 1 nM to 10 μM for BPA or BPA-G and (ii) from 1 pM to 10 nM for E2 or (iii) DMSO (solvent) alone. Final concentrations were obtained by adding 2 μL of the test chemical to 2 mL of the medium. Following incubation with the sample for 24 h, cells were harvested and assayed for luciferase activity according to the method described previously (*32*).

In the transiently transfected MCF-7 cells, the reference plasmid pCMV-lacZ was cotransfected as an internal control to correct for differences in transfection efficiencies and extraction differences. β-Galactosidase activity was measured according to standard protocols (*31*). Each treatment was carried out in duplicate, and two samples were taken from each replicate. Each experiment was repeated three times. Values are reported as a percentage relative to the maximum induction observed with 10 nM E2.

Uterotrophic Assay. The protocol for the uterotrophic assay has been previously described (*26*, *33*). Animals were treated by either gavage or sc injection with BPA suspended in arachis (peanut) oil. Clinical observations and body weights were recorded daily. The dosing volume used in both studies was 5 mL/kg of body weight, calculated on the weight of the animal immediately prior to administration. Animals were treated with BPA for three consecutive days and were killed by an overdose of halothane Ph Eur vapor approximately 24 h after the final dose. The dose levels for the oral gavage study were 0.002, 0.02, 0.2, 1.0, 10, 100, 200, and 800 mg of BPA/kg/day with 10 animals per treatment group. The dose levels for the sc injection study were 0.002, 0.02, 0.2, 1.0, 10, 100, and 800 mg of BPA/kg/day with 10 animals per treatment group. The high dose levels for both studies were based on preliminary work which established that oral and subcutaneous doses of up to 800 mg of BPA/kg/day did not induce more than mild toxicity in the experimental animals. The remaining dose levels were chosen by the BPA Global Industry Group to cover a wide range of exposure to the experimental animal. E2 was used as the positive control for both studies, using a dose level of 0.4 mg/kg of body weight/day, as recommended previously (*26*).

At necropsy, the uteri were excised, trimmed free of fat and any adhering nonuterine tissue, pierced, and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horn and the ovaries. The uterus was then weighed (wet weight), prior to bisection of the uterus at the junction of the uterine horns. One uterine horn was reweighed (wet) and then dried in a preweighed glass vial for a minimum of 24 h in an oven at a temperature of 70 °C. After being dried, the sample was removed and left to cool in a desiccator for a minimum of 1 h and the uterine dry weight recorded. The other section of the uterus was preserved in 10% neutral buffered formol saline and processed for histopathological examination. Liver weight and plasma alanine aminotransferase and aspartate aminotransferase levels were recorded, but the data are not presented in this paper.

Statistical analysis was conducted using the SAS (1989) package as follows: body weights, by analysis of covariance on initial body weight; and uterus weight, by analysis of variance and analysis of covariance on terminal body weights.

Results

Competitive Binding Ability of BPA and BPA-G. The ability of BPA and BPA-G to compete with [^{3}H]E2 for binding to mouse uterine ER, GST−hERαdef, and commercially available baculovirus-expressed full-length hERβ (Figure 1) preparations was investigated in vitro using a semi-high-throughput competitive binding assay. BPA competed for binding to each of the ER preparations; however, the IC_{50} values varied among preparations (Table 1). BPA bound to hERβ approximately 27- and 38-fold better than GST−hERαdef and mouse uterine cytosol, respectively (Table 1). BPA-G did not competitively displace [^{3}H]E2 from any of the ER preparations (Figure 1) at the concentrations that were examined (1 nM to 100 μM). Concentrations of > 100 μM were not examined due to possible artifactual results from competitor ligand precipitation.

Ability of BPA and BPA-G To Induce ERα- and ERβ-Mediated Gene Expression. BPA and BPA-G

Table 1. Summary of the IC_{50} Values of the Competitive Binding of 17β-Estradiol, Bisphenol A, and Bisphenol A Glucuronide to GST–hERαdef, Recombinant hERβ, and Mouse Uterine Cytosol

chemical	IC_{50} (M)[a]		
	GST–hERαdef	hERβ	mouse cytosol
17β-estradiol	$(2.9 \pm 0.5) \times 10^{-9}$	$(3.6 \pm 0.4) \times 10^{-9}$	$(3.0 \pm 0.2) \times 10^{-9}$
bisphenol A	$(3.6 \pm 1.6) \times 10^{-5}$	$(9.6 \pm 2.3) \times 10^{-7}$	$(2.6 \pm 1.1) \times 10^{-5}$
bisphenol A glucuronide	nb[b]	nb	nb

[a] IC_{50} values represent the means and standard deviations from three independent experiments. [b] nb denotes nonbinder since this chemical did not displace more than 10% of the [^{3}H]E2 at the highest concentration that was examined (100 μM).

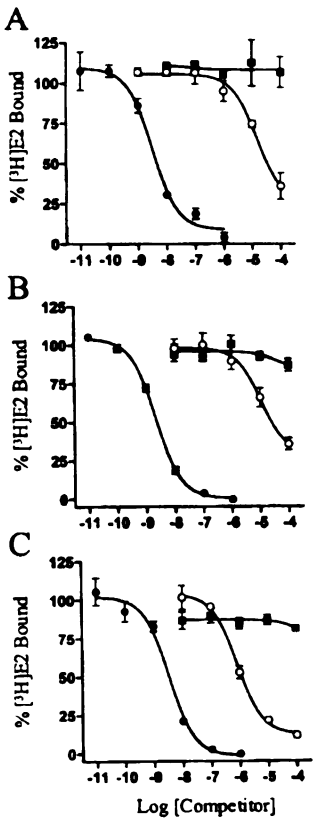

Figure 1. Competitive binding of BPA and BPA glucuronide to (A) the mouse uterine ER, (B) GST–hERαdef, and (C) recombinant hERβ. Protein preparations were incubated with 2.5 nM [^{3}H]E2 and increasing concentrations of unlabeled (●) E2, (○) BPA, or (■) BPA-G. The competitive displacement of radioligand was assessed using a vacuum pump filteration method as described in Experimental Procedures. Displacement curves were obtained for E2 and BPA. No significant displacement was observed with BPA-G. The data are the results from a representative experiment that was repeated three times.

induction of ER-mediated gene expression was assessed by measuring luciferase activity using MCF-7 cells cotransfected with Gal4-hERαdef or Gal4-hERβdef and the Gal4-regulated luciferase reporter gene, 17m5-G-Luc. The results in Figure 2 show that E2 treatment of transiently transfected MCF-7 cells with either Gal4-hERαdef or Gal4-hERβdef caused a concentration-dependent increase in luciferase activity. The EC_{50} values for this response were similar for both chimeric receptors (Table 2). MCF-7 cells transiently transfected with Gal4-hERαdef and Gal4-hERβdef exhibited a maximum induction response of approximately 30- and 20-fold, respectively, following treatment with 10 nM E2.

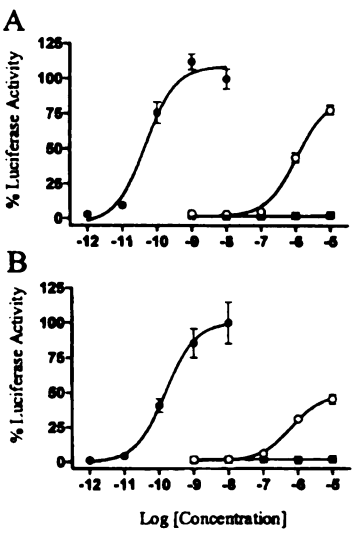

Figure 2. Effect of BPA and BPA glucuronide on reporter gene expression. MCF-7 cells transiently transfected with Gal4-human estrogen receptor α (Gal4-hERαdef) or Gal4-human estrogen receptor β (Gal4-hERβdef) and the Gal4-regulated reporter gene (17m-5-G-Luc) were treated with (●) E2, (○) BPA, or (■) BPA-G. Luciferase activity was measured 24 h after treatment as described in Experimental Procedures. The data are the results from a representative experiment that was repeated three times.

Table 2. Summary of the Ability of 17β-estradiol, Bisphenol A, and Bisphenol A Glucuronide to Induce Estrogen Receptor-mediated Gene Expression

chemical	EC_{50} (M)[a]	
	Gal4-hERαdef	Gal4-hERβdef
17β-estradiol	$(5.3 \pm 2.1) \times 10^{-11}$	$(8.3 \pm 2.9) \times 10^{-11}$
bisphenol A	$(7.1 \pm 2.9) \times 10^{-7}$	$(4.5 \pm 1.8) \times 10^{-7}$
bisphenol A glucuronide	ni[b]	ni

[a] EC_{50} values represent the means and standard deviations from three independent experiments. [b] ni denotes no induction.

BPA caused a dose-dependent increase in luciferase activity in MCF-7 cells transiently transfected with either Gal4-hERαdef or Gal4-hERβdef (Figure 2). The EC_{50} values for the response were similar for both ERα and ERβ (Table 2). Concentrations of >10 μM were not examined due to visible precipitation in the medium. BPA-G did not significantly induce ERα- or ERβ-mediated luciferase activity at the highest concentration that was examined (10 μM) (Figure 2). Similar results were observed using a Gal4-mERβdef consutruct (data not shown). Induction of luciferase activity by BPA was absent in transiently transfected MCF-7 cells if the chimeric construct, Gal4-hERαdef or Gal4-hERβdef, was not cotransfected (data not shown).

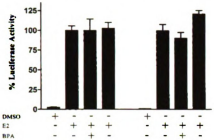

Figure 3. Effect of BPA and BPA-G cotreatment on E2 induction of reporter gene activity. MCF-7 cells transiently transfected with Gal4-human estrogen receptor α (Gal4-hERαdef) or Gal4-human estrogen receptor β (Gal4-hERβdef) and the Gal4-regulated reporter gene (17m-5-G-Luc) were cotreated with 10 nM E2 and 10 μM BPA or 10 μM BPA-G. The data are the results from a representative experiment that was repeated two times.

Figure 3 illustrates that cotreatment of MCF-7 cells with 10 μM BPA or BPA-G did not result in significant changes in the E2 induction of luciferase activity in MCF-7 cells transiently transfected with either Gal4-hERαdef or Gal4-hERβdef.

Uterine Effects following Oral Administration of BPA. There was a significant increase in mean uterine wet and dry weight in the groups treated with 200 and 800 mg of BPA/kg/day in comparison with the vehicle control group (Table 3). As expected, the uterine wet and dry weights in the positive control group were significantly increased compared with those of the vehicle control group.

Minimal to moderate endometrial hypertrophy/hyperplasia was observed in eight of the females treated with 800 mg of BPA/kg/day (Table 4). This effect was similar to, but less pronounced than, that of the positive control group (treated with 0.4 mg of E2/kg/day), where all the females exhibited moderate or marked change. Dose-related increases in severity of endometrial glandular epithelial apoptosis and the number of stromal neutrophils were observed in females treated with 200 or 800 mg of BPA/kg/day and also in the positive control group animals, where the increases were greater than those observed in the BPA-treated animals. An increased incidence and severity of luminal epithelial apoptosis was also observed in females treated with 800 mg of BPA/kg/day, although this was not observed in the positive control group. There were no treatment-related microscopic changes observed in the uteri of the animals from the remaining groups.

One female in the group treated with 800 mg of BPA/kg/day was killed humanely due to adverse clinical signs, although there were no abnormalities observed at necropsy that could explain these signs. There was no effect of treatment on body weight gain.

Uterine Effects following Subcutaneous Injection of BPA. There was a slight but significant reduction in normalized body weight over the treatment period in the

Table 3. Effect of Oral Gavage of Bisphenol A on Blotted and Dry Uterine Weight in Rats

	dose level (mg/kg/day)	no. of animals	terminal body weight (g) (mean ± SD)	uterus weight (mg) (mean ± SD)	½ uterus blotted weight (mg) (mean ± SD)	½ uterus dry weight (mg) (mean ± SD)	½ uterus percent loss on drying (mean ± SD)
arachis oil	0	10	61.7 ± 4.5	29 ± 9	13 ± 3	3.4 ± 0.04	73.3 ± 3.8
bisphenol A	0.002	10	61.2 ± 6.1	34 ± 8	16 ± 3	3.8 ± 0.09	75.9 ± 2.2
bisphenol A	0.02	10	59.8 ± 6.3	31 ± 9	15 ± 3	3.8 ± 0.06	73.4 ± 3.6
bisphenol A	0.2	10	60.5 ± 8.4	31 ± 9	15 ± 4	3.7 ± 0.12	75.4 ± 3.1
bisphenol A	1	10	61.2 ± 6.6	33 ± 9	16 ± 4[a]	4.3[a] ± 0.11	73.7 ± 5.2
bisphenol A	10	10	61.8 ± 6.3	30 ± 11	13 ± 3	3.7 ± 0.09	71.8 ± 5.5
bisphenol A	100	10	60.6 ± 6.9	29 ± 6	14 ± 3	3.6 ± 0.08	74.0 ± 3.0
bisphenol A	200	10	61.8 ± 5.5	36 ± 8[a]	17 ± 4[a]	4.3 ± 0.07[a]	74.4 ± 2.2
bisphenol A	800	9	60.7 ± 6.7	58 ± 14[b]	28 ± 6[b]	6.0 ± 0.08[b]	78.1 ± 2.6[b]
17β-estradiol	0.4	10	61.4 ± 5.1	109 ± 19[b]	52 ± 11[b]	10.3 ± 0.18[b]	80.0 ± 1.1[b]

[a] $p < 0.05$ with a Student's t test (two-sided). [b] $p < 0.01$ with a Student's t test (two-sided).

Table 4. Microscopic Changes in the Uterus following Oral Gavage of Bisphenol A

	0[a]	0.002[a]	0.02[a]	0.2[a]	1[a]	10[a]	100[a]	200[a]	800[a]	0.4[b]
no. of animals	10	10	10	10	10	10	10	10	9	10
endometrial hypertrophy/hyperplasia										
minimal	0	0	0	0	0	0	0	2	4	0
slight	0	0	0	0	0	0	0	0	3	0
moderate	0	0	0	0	0	0	0	0	1	8
marked	0	0	0	0	0	0	0	0	0	2
endometrial epithelial apoptosis (lumen)										
minimal	1	0	0	1	0	0	1	0	3	3
slight	0	0	0	0	0	0	0	1	2	0
moderate	0	0	0	0	0	0	0	0	1	0
endometrial epithelial apoptosis (glands)										
minimal	7	5	4	8	4	5	6	6	3	0
slight	0	0	0	0	0	0	0	2	1	1
moderate	0	0	0	0	0	0	0	0	3	8
increased stromal neutrophils										
minimal	0	0	0	0	0	0	0	2	4	0
slight	0	0	0	0	0	0	0	0	4	2
moderate	0	0	0	0	0	0	0	0	0	8

[a] Milligrams of BPA per kilogram of body weight per day. [b] Milligram of E2 per kilogram of body weight per day.

Table 5. Effect of Subcutaneous Administration of Bisphenol A on Blotted and Dry Uterine Weight in Rats

	dose level (mg/kg/day)	no. of animals	terminal body weight (g) (mean ± SD)	uterus weight (mg) (mean ± SD)	½ uterus blotted weight (mg) (mean ± SD)	½ uterus dry weight (mg) (mean ± SD)	½ uterus percent loss on drying (mean ± SD)
arachis oil	0	10	63.3 ± 2.4	42 ± 8	19 ± 5.1	2.9 ± 1.7	85.3 ± 6.7
bisphenol A	0.002	10	61.9 ± 4.5	41 ± 14	19 ± 5.6	3.0 ± 2.7	85.2 ± 13.4
bisphenol A	0.02	10	61.4 ± 3.4	48 ± 21	22 ± 11	3.3 ± 4.7	88.7 ± 10.5
bisphenol A	0.2	10	63.4 ± 6.4	37 ± 8	16 ± 3.3	1.6 ± 1.4	90.1 ± 7.8
bisphenol A	1	10	66.5 ± 3.5	43 ± 10	21 ± 4.2	3.0 ± 2.4	86.7 ± 9.1
bisphenol A	10	10	66.4 ± 1.7	44 ± 10	19 ± 4.9	2.0 ± 2.1	89.5 ± 12.8
bisphenol A	100	10	64.4 ± 2.4	51 ± 5	22 ± 3.7	1.2 ± 1.2	94.9 ± 4.7[a]
bisphenol A	800	10	62.6 ± 4.0	91 ± 33[b]	42 ± 14	4.3 ± 3.7	90.7 ± 6.0
17β-estradiol	0.4	10	62.4 ± 5.8	135 ± 14[b]	60 ± 6.4[b]	7.7 ± 2.4[b]	87.5 ± 3.4

[a] $p < 0.05$ with a Student's t test (two-sided). [b] $p < 0.01$ with a Student's t test (two-sided).

Table 6. Microscopic Changes in the Uterus following Subcutaneous Administration of Bisphenol A

	0[a]	0.002[a]	0.02[a]	0.2[a]	1[a]	10[a]	100[a]	800[a]	0.4[b]
no. of animals	10	10	10	10	10	10	10	10	10
endometrial hypertrophy/hyperplasia									
minimal	0	0	0	0	0	0	8	0	0
slight	0	0	0	0	0	0	0	1	0
moderate	0	0	0	0	0	0	0	8	2
marked	0	0	0	0	0	0	0	1	8
endometrial epithelial apoptosis (lumen)									
minimal	0	0	0	0	0	6	1	4	0
slight	0	0	0	0	0	0	9	0	0
moderate	0	0	0	0	0	0	0	2	0
endometrial epithelial apoptosis (glands)									
minimal	0	0	0	0	0	6	1	1	3
slight	0	0	0	0	0	0	9	3	3
moderate	0	0	0	0	0	0	0	5	1
increased stromal neutrophils									
minimal	0	0	0	0	0	0	10	2	0
slight	0	0	0	0	0	0	0	8	1
moderate	0	0	0	0	0	0	0	0	9
vacuolar degeneration of the endometrial epithelium									
minimal	0	0	0	0	0	0	0	0	5
slight	0	0	0	0	0	0	0	0	2
mononuclear cell infiltration (total)	0	0	0	0	0	0	1	0	0
minimal	0	0	0	0	0	0	1	0	0

[a] Milligrams of BPA per kilogram of body weight per day. [b] Milligram of E2 per kilogram of body weight per day.

group treated with 800 mg of BPA/kg/day and in the positive control group in comparison with the control group (data not shown).

There was a significant increase in mean uterine wet and dry weights in the group treated with 800 mg of BPA/kg/day, compared with those in the vehicle control group (Table 5). The mean uterine wet weight for the group treated with 100 mg of BPA/kg/day was also increased, but was not statistically significance. As expected, the uterine wet and dry weights in the positive control group were significantly increased compared with those of the vehicle control group.

Slight to marked endometrial hypertrophy/hyperplasia was observed in all females treated with 800 mg of BPA/kg/day (Table 6). This effect was similar to, but less pronounced than, that of the positive control group (treated with 0.4 mg of E2/kg/day). At 100 mg of BPA/kg/day, eight females were observed with minimal change. Increases in the severity of endometrial glandular epithelial apoptosis were observed in females treated with 10, 100, or 800 mg of BPA/kg/day. These findings were also observed in the positive control group animals, where the increase and severity were lower than those observed in the animals treated with 800 mg of BPA. Increases in the number of stromal neutrophils were observed in females treated with 100 or 800 mg of BPA/kg/day. These findings were also observed in the positive control group animals, where the increases were higher than those observed in the animals treated with 800 mg of BPA. Luminal epithelial apoptosis was observed in females treated with 10, 100, and 800 mg of BPA/kg/day, with the highest incidence occurring in the group treated with 100 mg of BPA, although this was not observed in the positive control group. There were no treatment-related microscopic changes observed in the uteri of the animals from the remaining groups. There were no clinical signs observed during the study that were considered to be related to treatment.

Discussion

The ligand binding affinities of ERα and ERβ proteins for physiological ligands, such as E2, are quite similar (*12*). However, differences in ligand preference and relative binding affinity between subtypes for other estrogenic chemicals and phytoestrogens have been demonstrated (*11*, *34*, *35*) and were observed in this study. BPA exhibited a 38- and 27-fold greater ability to compete for binding to ERβ than to GST−hERαdef and mouse uterine cytosol, respectively. This differential binding is comparable to previously reported IC_{50} values (*12*), although lower values have been reported for other ERβ preparations (*11*). The reported differences may be due to the use of a baculovirus/SF9-expressed ERβ preparation in the current study that included an ad-

ditional 53 amino acid residues at the N-terminus (*36*), which was absent from earlier clones (*10*, *37*) and not present in the ERβ protein preparations used in other binding studies (*11*, *12*). Nevertheless, the amino acid sequences within the respective ligand binding domains are identical in all of the competitive binding studies. Differences in reported ERβ IC_{50} values for the same ligand among studies may also be due to variation in assay protocols. In contrast, BPA-G did not compete with [^{3}H]E2 for binding to either protein preparation.

Other studies have also reported differences in the relative binding affinity between ERα and ERβ for the same compound. For example, an aryl-substituted pyrazole exhibited higher binding affinity for ERα and 120-fold greater potency in transactivational activity than ERβ (*35*). In addition, genistein has been shown to preferentially bind with 30-fold greater potency to hERβ than to hERα (*12*). The overall structure of the ERβ ligand binding pocket is similar to that of ERα with ligand binding being achieved by a combination of specific hydrogen bonding interactions and the hydrophobic nature of the binding pocket (*38*–*40*). It has been suggested that the preference for genistein may be attributed to two conservative mutations within the binding pocket that further stabilize the hERβ–genistein complex (*38*).

BPA has been shown to exhibit mixed agonist and antagonistic effects through ERα while behaving solely as an agonist through ERβ (*41*). However, in the current study, no antagonistic effects were observed. Despite the preferential binding for hERβ, BPA exhibited an activity in Gal4-hERβdef reporter gene induction that was <2-fold greater than the activity in Gal4-hERαdef. The lack of correlation between binding affinity and transactivational activity may be due, in part, to alterations in the ability of the liganded ER complex to bind DNA (*42*, *43*). Differential interactions between ERα and ERβ with cellular proteins, such as coactivators, may also be a contributing factor (*44*). Crystal structure data for ERβ complexed with genistein indicate that helix 12, which interacts with coactivators, is positioned along a cleft rather than over the binding cavity as seen ERα–E2 and ERα–diethylstilbestrol structures (*39*, *45*). Therefore, the ligand-dependent positioning of helix 12 may account for the difference in the ability of genistein to induce ERα- and ERβ-mediated gene expression, despite the greater affinity for ERβ. To date, no ERα–genistein structure has been reported to support this hypothesis.

In vivo effects of BPA on uterine weight and uterine microscopic changes were examined in immature rats treated by gavage to mimic the primary route of exposure, and by sc to bypass hepatic first-pass elimination and subsequent metabolism to BPA-G. Increases in wet and dry uterine weight were observed at doses of 800 mg of BPA/kg/day for administration by both routes of exposure and also at a dose of 200 mg of BPA/kg/day for oral administration. The effects on uterine weight are consistent with other recent reports (*46*, *47*). However, rat strain differences in vaginal responses to BPA have been reported (*48*). In addition, sc administration of BPA has been shown to increase uterine wet weight in B6C3F1 mice at doses ranging from 0.02 to 8 mg/day (*49*). EC_{50} values of 0.72 mg of BPA/day and 19.4 ng of E2/day have been estimated for this response (*49*), although it has been argued that these values do not represent true potency comparisons, since BPA-induced a <2-fold increase in uterine weight compared to a >5-fold increase induced by E2 (*50*). Tinwell and co-workers (*51*) were unable to demonstrate that BPA is reproducibly active in the mouse uterotrophic assay in which AP mice were treated with either by sc or gavage with doses ranging from 0.02 μg/kg to 300 mg/kg of BPA. This is also consistent with the work of Coldham et al. (*52*), where sc injection of BPA failed to increase uterine weight at dose levels of 3, 33, and 330 mg/kg.

Increases in the incidence of endometrial hypertrophy, endometrial epithelial apoptosis in the lumen, and endometrial and stromal neutrophils were observed in gavaged rats treated with 200 and 800 mg of BPA/kg/day. Subcutaneous injections of 10, 100, and 800 mg of BPA/kg/day also increased the incidence of changes in the microscopic structure of the uterus. These microscopic findings are similar to effects induced by other weak estrogen agonists (*53*) and are consistent with vaginal cornification results (*47*).

BPA has also been shown to induce molecular, cellular, and tissue level effects in rats at doses that did not induce uterine weight. BPA induced progesterone receptor expression in rats treated with 5–150 mg/kg (*21*). Similarly, BPA and the phytoestrogen, diadzin, were weak stimulators of uterine growth in rat, but were able to alter the expression of the androgen receptor, the estrogen receptor, and complement 3 (*54*). Therefore, weak estrogenic chemicals can induce changes at the gene expression and cellular levels without affecting uterine weight. However, the toxicological significance of these changes is unclear.

At low doses that do not induce uterine wet weight, gestational exposure of mice to BPA has been reported to affect accessory reproductive organ weights and daily sperm production in male offspring (*55*, *56*). In contrast, other rodent studies indicate that 0.2–200 μg of BPA/kg/day in drinking water does not affect reproductive organ development or sperm counts (*57*, *58*). These contradictory results observed in similar assays make the assessment of BPA and other xenoestrogens a contentious issue that needs to be resolved (*50*).

The results of this study demonstrate that BPA exhibits a greater ability to compete for binding to ERβ than to ERα. However, the ability of BPA to induce reporter gene expression mediated by either isotype is comparable. In addition, these results demonstrate that there is a clear route of administration dependency on the ability of BPA to induce uterine responses, as observed in Long Evans rats (*59*). This route dependency supports results from competitive binding and reporter gene assays that demonstrate the BPA-G does not exhibit significant estrogenic activity in the uterus, since BPA is more rapidly metabolized to BPA-G when administered by gavage than when administered by sc injection (*23*).

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References

(1) Safe, S. H. (2000) Endocrine Disruptors and Human Health-Is There a Problem? An Update. *Environ. Health Perspect.* **108**, 487–493.

(2) Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1995) Declining semen quality and increasing incidence of testicular cancer: is there a common cause? *Environ. Health Perspect.* **103**, 137–139.

(3) Sharpe, R. M., and Skakkebaek, N. E. (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* **341**, 1392–1395.
(4) Irvine, D. S. (1994) Falling sperm quality. *Br. Med. J.* **309**, 476.
(5) Reijnders, P. J. (1986) Reproductive failure in common seals feeding on fish from polluted coastal waters. *Nature* **324**, 456–457.
(6) Guillette, L. J. J., Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F., and Woodward, A. R. (1994) Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* **102**, 680–688.
(7) Fry, D. M. (1995) Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ. Health Perspect.* **103**, 165–171.
(8) Von Westernhagen, H., Rosenthal, H., Dethlefsen, V., Ernst, W., Harms, U. L., and Hansen, P. D. (1981) Bioaccumulating substances and reproductive success in the Baltic flounder, *Platichthys flesus*. *Aquat. Toxicol.* **1**, 85–99.
(9) Evans, R. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889–895.
(10) Mosselman, S., Polman, J., and Dijkema, R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **392**, 49–53.
(11) Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139**, 4252–4263.
(12) Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. A. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863–870.
(13) Tenbaum, S., and Baniahmad, A. (1997) Nuclear receptors: structure, function and involvement in disease. *Int. J. Biochem. Cell Biol.* **29**, 1325–1341.
(14) Webster, N. J., Green, S., Jin, J. R., and Chambon, P. (1988) The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. *Cell* **54**, 199–207.
(15) White, R., Fawell, S. E., and Parker, M. G. (1991) Analysis of oestrogen receptor dimerisation using chimeric proteins. *J. Steroid Biochem. Mol. Biol.* **40**, 333–341.
(16) Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J.* **11**, 1025–1033.
(17) Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279–2286.
(18) Smeets, J. M., van Holsteijn, I., Giesy, J. P., Seinen, W., and van den Berg, M. (1999) Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. *Toxicol. Sci.* **50**, 206–213.
(19) Chun, T. Y., and Gorski, J. (2000) High concentrations of bisphenol A induce cell growth and prolactin secretion in an estrogen-responsive pituitary tumor cell line. *Toxicol. Appl. Pharmacol.* **162**, 161–165.
(20) Morrissey, R. E., George, J. D., Price, C. J., Tyl, R. W., Marr, M. C., and Kimmel, C. A. (1987) The developmental toxicity of bisphenol A in rats and mice. *Fundam. Appl. Toxicol.* **8**, 571–582.
(21) Gould, J. C., Leonard, L. S., Maness, S. C., Wagner, B. L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D. P., and Gaido, K. W. (1998) Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol. Cell. Endocrinol.* **142**, 203–214.
(22) Knaak, J. B., and Sullivan, L. J. (1966) Metabolism of bisphenol A in the rat. *Toxicol. Appl. Pharmacol.* **8**, 175–184.
(23) Pottenger, L. H., Domoradzki, J. Y., Markham, D. A., Hansen, S. C., Cagen, S. Z., and Waechter, J. M., Jr. (2000) The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. *Toxicol. Sci.* **54**, 3–18.
(24) Brotons, J. A., Olea-Serrano, M. F., Villalobos, M., Pedraza, V., and Olea, N. (1995) Xenoestrogens released from lacquer coatings in food cans. *Environ. Health Perspect.* **103**, 608–612.
(25) Olea, N., Pulgar, R., Perez, P., Olea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A. M., and Sonnenschein, C. (1996) Estrogenicity of resin-based composites and sealants used in dentistry. *Environ. Health Perspect.* **104**, 298–305.
(26) Odum, J., Lefevre, P. A., Tittensor, S., Paton, D., Routledge, E. J., Beresford, N. A., Sumpter, J. P., and Ashby, J. (1997) The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. *Regul. Toxicol. Pharmacol.* **25**, 176–188.
(27) Matthews, J. B., and Zacharewski, T. R. (2000) Differential binding affinities of PCBs, HO-PCBs and Aroclors with recombinant human, rainbow trout (*Onchorhynkiss mykiss*) and reptilian (*Anolis carolinensis*) estrogen receptors using a semi-high throughput competitive binding assay. *Toxicol. Sci.* **53**, 326–339.
(28) Gillesby, B., and Zacharewski, T. R. (1999) pS2 (TFF2) mRNA levels in human breast tumor samples: correlation with clinical and histological prognostic markers. *Breast Cancer Res. Treat.* **56**, 253–265.
(29) Balaguer, P., Joyeux, A., Denison, M. S., Vincent, R., Gillesby, B. E., and Zacharewski, T. (1996) Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using in vitro recombinant receptor-reporter gene assays. *Can. J. Physiol. Pharmacol.* **74** (2), 216–222.
(30) Zacharewski, T. R., Meek, M. D., Clemons, J. H., Wu, Z. F., Fielden, M. F., and Matthews, J. B. (1998) Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalate esters. *Toxicol. Sci.* **46**, 282–293.
(31) Sambrook, J., Fristch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
(32) Brasier, A. R., Tate, J. E., and Habener, J. F. (1989) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *BioTechniques* **7**, 1116–1122.
(33) Wakeling, A. E., Dukes, M., and Bowler, J. (1991) A potent specific pure antiestrogen with clinical potential. *Cancer Res.* **51**, 3867–3873.
(34) Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J., and Nilsson, S. (1998) Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol. Pharmacol.* **54**, 105–112.
(35) Sun, J., Meyers, M. J., Fink, B. E., Rajendran, R., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1999) Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-alpha or estrogen receptor-beta. *Endocrinology* **140**, 800–804.
(36) Ogawa, S., Inoue, S., Watanabe, T., Hiroi, H., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998) The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. *Biochem. Biophys. Res. Commun.* **243**, 122–126.
(37) Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5925–5930.
(38) Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J., and Carlquist, M. (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* **18**, 4608–4618.
(39) Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**, 753–758.
(40) Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5998–6003.
(41) Hiroi, H., Tsutsumi, O., Momoeda, M., Takai, Y., Osuga, Y., and Taketani, Y. (1999) Differential interactions of bisphenol A and 17β-estradiol with estrogen receptor α (ERα) and ERβ. *Endocr. J.* **46**, 773–778.
(42) Christman, J. K., Nehls, S., Polin, L., and Brooks, S. C. (1995) Relationship between estrogen structure and conformational changes in estrogen receptor/DNA complexes. *J. Steroid Biochem. Mol. Biol.* **54**, 201–210.
(43) Pennie, W. D., Aldridge, T. C., and Brooks, A. N. (1998) Differential activation by xenoestrogens of ER alpha and ER beta when linked to different response elements. *J. Endocrinol.* **158**, R11–R14.
(44) Paige, L. A., Christensen, D. J., Gron, H., Norris, J. D., Gottlin, E. B., Padilla, K. M., Chang, C. Y., Ballas, L. M., Hamilton, P. T., McDonnell, D. P., and Fowlkes, D. M. (1999) Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3999–4004.
(45) Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) The structural basis of

estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**, 927–937.

(46) Ashby, J., and Tinwell, H. (1998) Uterotrophic activity of bisphenol A in the immature rat. *Environ. Health Perspect.* **106**, 719–720.

(47) Ashby, J., Odum, J., Paton, D., Lefevre, P. A., Beresford, N., and Sumpter, J. P. (2000) Re-evaluation of the first synthetic estrogen, 1-keto-1,2,3,4-tetrahydrophenanthrene, and bisphenol A, using both the ovariectomised rat model used in 1933 and additional assays. *Toxicol. Lett.* **115**, 231–238.

(48) Long, X., Steinmetz, R., Ben-Jonathan, N., Caperell-Grant, A., Young, P. C., Nephew, K. P., and Bigsby, R. M. (2000) Strain differences in vaginal responses to the xenoestrogen bisphenol A. *Environ. Health Perspect.* **108**, 243–247.

(49) Papaconstantinou, A. D., Umbreit, T. H., Fisher, B. R., Goering, P. L., Lappas, N. T., and Brown, K. M. (2000) Bisphenol A-induced increase in uterine weight and alterations in uterine morphology in ovariectomized B6C3F1 mice: role of the estrogen receptor. *Toxicol. Sci.* **56**, 332–339.

(50) Safe, S. (2000) Bisphenol A and related endocrine disruptors. *Toxicol. Sci.* **56**, 251–252.

(51) Tinwell, H., Joiner, R., Pate, I., Soames, A., Foster, J., and Ashby, J. (2000) Uterotrophic activity of bisphenol A in the immature mouse. *Regul. Toxicol. Pharmacol.* **32**, 118–126.

(52) Coldham, N. G., Dave, M., Sivapathasundaram, S., McDonnell, D. P., Connor, C., and Sauer, M. J. (1997) Evaluation of a recombinant yeast cell estrogen screening assay. *Environ. Health Perspect.* **105**, 734–742.

(53) Branham, W. S., Zehr, D. R., and Sheehan, D. M. (1993) Differential sensitivity of rat uterine growth and epithelium hypertrophy to estrogens and antiestrogens. *Proc. Soc. Exp. Biol. Med.* **203**, 297–303.

(54) Diel, P., Schulz, T., Smolnikar, K., Strunck, E., Vollmer, G., and Michna, H. (2000) Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotropic activity. *J. Steroid Biochem. Mol. Biol.* **73**, 1–10.

(55) Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M., and Welshons, W. V. (1997) Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol [see comments]. *Environ. Health Perspect.* **105**, 70–76.

(56) vom Saal, F. S., Cooke, P. S., Buchanan, D. L., Palanza, P., Thayer, K. A., Nagel, S. C., Parmigiani, S., and Welshons, W. V. (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol. Ind. Health* **14**, 239–260.

(57) Cagen, S. Z., Waechter, J. M., Jr., Dimond, S. S., Breslin, W. J., Butala, J. H., Jekat, F. W., Joiner, R. L., Shiotsuka, R. N., Veenstra, G. E., and Harris, L. R. (1999) Normal reproductive organ development in Wistar rats exposed to bisphenol A in the drinking water. *Regul. Toxicol. Pharmacol.* **30**, 130–139.

(58) Cagen, S. Z., Waechter, J. M., Jr., Dimond, S. S., Breslin, W. J., Butala, J. H., Jekat, F. W., Joiner, R. L., Shiotsuka, R. N., Veenstra, G. E., and Harris, L. R. (1999) Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. *Toxicol. Sci.* **50**, 36–44.

(59) Laws, S. C., Carey, S. A., Ferrell, J. M., Bodman, G. J., and Cooper, R. L. (2000) Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. *Toxicol. Sci.* **54**, 154–167.

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CHAPTER 8

Purification and characterization of the rtERα D, E, and F domains

Abstract

The rainbow trout estrogen receptor α (rtERα) and human ERα (hERα) have different functional activities in response to 17β-estradiol (E2). Sequence alignment has identified two amino acid residues that line their respective ligand binding pockets and differ between the rtERα (Met317, Ile496) and hERα (Leu349, Met528). In the rtERα, these residues have been previously shown to influence E2 binding and transactivation ability. In an effort to design an efficient expression and purification strategy to generate sufficient amounts of protein for further biophysical characterization of the rtERα, a series of recombinant proteins consisting of combinations of the D, E, and F domains fused to either glutathione-S-transferase (GST) (GST-rtERα) or a 6x histidine tag (his_rtERα) were examined. Although both expression systems produced protein with similar activity, the 6x histidine tagged approach resulted in increased yield and purity. In both expression systems the addition of the hydrophilic D domain improved protein solubility, whereas the F domain did not appear to have any effect. After consideration of yield, purity, and activity, the his_rtERα DE protein was chosen for initial crystallographic screens. Unfortunately, two successive screens have not produced any crystals. A more thorough purification strategy using ion exchange and/or size exclusion chromatography maybe required. These data demonstrate that the D domain can significantly improve the solubility of the rtERα. In addition, we have developed a robust expression and purification strategy that can yield sufficient amounts of protein for biophysical characterization of the rtERα.

Introduction

Nuclear magnetic resonance and x-ray diffraction studies of proteins are often limited by the amount of purified protein. Performing such studies on native proteins is limited because many proteins of interest are produced at very low levels within the cell. Heterologous expression systems circumvent this problem since they generally provide sufficient levels of proteins for these types of studies. Heterologous systems using Escherichia coli provide several advantages for protein expression since they have relative short doubling times and extensive knowledge exists about their genetic make up, biochemistry, and physiology. All of which can be tailored to a specific expression problem (1),(2). However, many post-translation modifications do not occur in E. coli, which may affect overall protein folding and consequently its function.

A comparison of the human α (hERα) and rainbow trout α rtERα) estrogen receptors indicates that the E domain is 60% identical in amino acid sequence (Chapter 3, figure 3A page 61), whereas the D and F domains are only 18% and 19% identical, respectively. Previous studies and data presented in chapter 6 show that the rtERα exhibits differential functional activities in response to E2 compared to hERα (3),(4). Sequence alignment has identified two amino acid substitutions within the ligand binding pocket that have been shown in chapter 6 to influence E2 mediated transactivation and the temperature sensitivity of the rtERα. However, in the absence of structural data of the rtERα-E2 complex, the role that these residues (M317 and I496) play in influencing the binding mode of E2 is unknown.

It has been reported that in the expression of GST-rtERα (CD) and GST-rtERα (EF) in E. coli nearly 100% of both fusion proteins were insoluble (5). The authors used

denaturing conditions to solubilize the protein followed by renaturation using gradual dialysis to verify receptor activity (5). This is in contrast to the data presented in chapter 3 where we describe satisfactory yields of a GST-rtERα DEF fusion protein using native conditions. This suggests that the different domains included in the fusion protein may influence its expression in E. coli.

Several nuclear receptors have been overexpressed in bacteria, purified and their structures determined. Although previous ER proteins were purified and subsequently crystallized using a customized E2-affinity column (6,7), it has been recently reported that his tagged hERα E domain wild-type and mutant proteins were crystallized from metal affinity chromatography purified protein (8). The overall structure of the wild-type hERα E domain was similar to previous reports (8). To date the structure of only the E domains of human and rodent ERs have been solved and these structures do not contain any information regarding the role that the D and F domains may play in influencing ligand binding and/or coactivator interaction. Determining the position of the F domain in the ER crystal structure may be of significant interest since it this region has been shown to influence coactivator recruitment (9), and is also poorly conserved among ERs from several vertebrate species.

To examine the potential role of the D and F domains on the solubility and functionality of the rtERα, as well as produce sufficiently pure protein to initiate crystallographic studies of the rtERα, a series of GST and histidine tagged fusion proteins were expressed in bacteria and purified using affinity chromatography in support of future crystallization trials.

Materials and Methods

Plasmid construction

The plasmid pGEX-rtERα DEF harboring the rainbow trout ERα D, E, and F domains (a.a. 225 to 577) was constructed as described in chapter 3. pGEX-rtERα E (a. a. 265 to 521), pGEX-rtERα EF (a. a. 265 to 577), and pGEX-rtERα DE (a. a. 225 to 521) were constructed by PCR amplification of the desired sequences of the rtERα using the following primer combinations rt1f (5'-caaaggatccggatggcgtgggcccagaatc -3') and rt2r (5'-caaactcgatggggattggagccggtgacc -3'), rt1f and rt3r (5'-aaaactcgagtcacggaatgggcatctggtctg -3'), and rt4f (5'-aaaaggatcccgggttctcaggaaggataagcg-3') and rt3r, respectively. The fragments were digested with the appropriate restriction enzymes and ligated into the similarly digested GST expression vector, pGEX6p3 (Amersham Pharmacia).

pEThis-rtERα E, pEThis-rtERα EF, pEThis-rtERα DE, and pEThis-rtERα DEF were constructed in a similar manner using the following PCR primer combinations rt5f and rt2r, rt5f (5'- caaaccatggatccacatcaccatcaccatcacctcaatggtggtggaggatggc -3') and rt3r, rt6f (5'- caaaccatggatccacatcaccatcaccatcaccgggttctcaggaaggataagcg -3') and rt2r, and rt6f and rt4r, respectively. For the construction of the pEThis-rtERα expression vectors, the 6x histidine sequence, which has been underlined above, was incorporated into the forward PCR primers creating an NH_2-terminal 6x histidine protein. The PCR fragments were digested with the appropriate restriction enzymes and ligated into the similarly digested pET23d(+) expression vector (Invitrogen), which contains a MDP leader sequence as described previously (7).

Expression and purification of GST-rtERα fusion proteins

E. coli strain BL21 (Amersham/Pharmacia) containing pGEX-rtERα constructs were grown in 2 x Luria broth (LB) and incubated at 30°C with constant shaking. Cells were induced with 1 mM IPTG at an optical density of 0.8 at 600 nm and grown for 4 h at 25°C. After centrifugation, cell pellets were resuspended in 15 ml of buffer A (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 50 mM NaCl and 10% (v/v) glycerol, pH 7.5) containing 0.1 mg/ml lysozyme, 200 U of Dnase I, and protease inhibitors. Cells were then lysed by sonication on ice and cell debris was pelleted by centrifugation at 20,000xg for 30 min at 4°C. The pellet was resuspended a second time and the supernatants pooled.

The supernatants containing the GST fusion proteins were incubated in batch with GSH Sepharose pre-equilibrated with buffer A (1 ml/15 ml supernatant) for 1.5 h at 4°C. After adsorption of the protein, the GSH Sepharose was applied to a 20 ml disposable column and washed with 50x bed volume of buffer A (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 200 mM NaCl and 10% (v/v) glycerol, pH 7.5), 50x bed volume of buffer B (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 1 M NaCl and 10% (v/v) glycerol, pH 7.5), and finally 10x bed volume of buffer A. Bound proteins were eluted in 25 ml of buffer C (50 mM HEPES, 3 mM EDTA, 5 mM DTT, 200 mM NaCl and 10% (v/v) glycerol, pH 8.0) containing 20 mM GSH. The partially purified protein was concentrated to a 1 ml final volume and the protein concentration was determined using the BioRad protein assay (BioRad).

Expression and purification of 6x histagged rtERα fusion proteins

E. coli strain BL21(DE3)pLysS (Invitrogen) containing pEThis-rtERα constructs were grown in 2x LB and incubated at 30°C with constant shaking. Cells were induced with 0.5 mM IPTG at an optical density of 0.8 at 600 nm and grown for 3 h at 25°C. After centrifugation, the pellets were resuspended in 20 ml of lysis buffer (20 mM Tris, 300 mM NaCl, 2 mM $MgCl_2$, 5 mM imidazole, 100 μg/ml PMSF, 5 mM β-mercaptoethanol, 200 U Dnase I, and 1 mg/ml lysozyme). Cells were then lysed by sonication on ice and cell debris was pelleted by centrifugation at 20,000xg for 30 min at 4°C. The pellet was resuspended a second time and the supernatants pooled.

The supernatants containing the his_rtERαs were incubated in batch with nickel nitrilotriacetic acid resin (Qiagen) pre-equilibrated with resuspension buffer (1 ml/40 ml supernatant) for 1.5 h at 4°C. After adsorption of the protein, the nickel affinity resin was applied to a 20 ml disposable column and washed with 50x bed volume of wash I (20 mM Tris, 500 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol), 50x bed volume of wash II (20 mM Tris, 1 M NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol), and finally 25x bed volume of wash III (20 mM Tris, 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol). Bound proteins were eluted in elution buffer (20 mM Tris, 300 mM NaCl, 5 mM imidazole, 100 mM EDTA) and collected as 1 ml fractions every 10 min. The protein concentration of each fraction was determined and fractions containing protein were pooled and the final concentration was determined using the BioRad protein assay (BioRad).

For the purification of the his_rtERα DE to be used in subsequent crystallization trials, 20 μM E2 was added to all buffers during the expression and purification steps. In addition, the protein was carboxymethylated in situ in the presence of 15 mM iodoacetic

acid in was III, followed by a 5x bed volume wash with wash III. The protein was then eluted as described above. The protein was dialyzed overnight against (20 mM Tris pH 7.4, 150 mM NaCl, 3 mM EDTA, 20 μM E2), concentrated to 7-10 mg/ml and stored at –80°C.

Receptor binding assays

Aliquots of crude and partially purified GST-rtERα and his_rtERα fusion proteins were diluted in TEGD buffer (10 mM Tris pH 7.6, 1.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol) containing 1 mg/ml bovine serum albumin as a carrier protein. The reactions were incubated 4°C for 24 h with a saturating amount of [^{3}H]E2 (3 nM) in 1 ml glass tubes arranged in a 96-well format (Marsh Scientific, Rochester, NY). Binding assays were initiated by adding 240 μl of protein preparation to glass tubes containing 5 μl of DMSO and 5 μl [^{3}H]E2; thus the concentration of solvent did not exceed 4 %. Bound [^{3}H]E2 was separated from free using a 96-well filter plate and vacuum pump harvester (Packard Instruments). After drying, the filter plates were sealed and 50 μl of MicroScint 20 scintillation cocktail (Packard Instruments) was added to each well. Bound [^{3}H]E2 was measured using a TopCount scintillation and luminescence counter (Packard Instruments).

Western Blotting

Purified GST-hERαdef samples (250 ng) were fractionated on a 10% polyacrylamide/SDS gel and transferred to nitrocellulose (Amersham/Pharmacia, Piscataway, NJ). Blots were incubated with anti-GST antibody (Santa Cruz, Santa Cruz,

CA) or anti-GroEL (Dr. Jon Kaguni, Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan) for 2 h at room temperature in the presence of 2% fat-free milk powder and then incubated for 2 h with the secondary conjugated goat anti-(mouse IgG) or mouse anti-(rabbit)-horseradish peroxidase (Santa Cruz). Proteins were visualized using ECL detection system (Amersham/Pharmacia) and subsequent exposure to film.

Results

Expression and purification of the GST-rtERα fusion proteins

The GST-rtERα fusion proteins migrated according to their predicted molecular weights (MWs): GST-rtERα E (55.6 kDa), GST-rtERα EF (61.3 kDa), GST-rtERα DE (60.0 kDa), and GST-rtERα DEF protein (65.7 kDa) (Figure 1). In addition, higher and lower MW proteins co-purified with the fusion proteins. Western blot analysis of partially purified GST-hERαDEF revealed that the contaminating band at approximately 60 kDa represents the bacterial chaperon protein GroEL (figure 2). GroEL has been reported to be a common copurifying contaminant of GST fusion proteins (10). The purity of the GST-rtERα fusion proteins varied among protein preparations and was estimated to be 60%-80%. The yields also varied, with GST-rtERα E, GST-rtERα EF, GST-rtERα DE, and GST-rtERα DEF yielding approximately 0.4, 2.5, 4.5, and 3.5 mg partially purified protein/L culture, respectively. In addition, only the GST-rtERα DE and GST-rtERα DEF proteins were visible in the crude proteins extracts, which have been denoted by an asteriks in Figure 1.

Receptor binding assays were performed on the crude and purified samples for each of the fusion proteins to evaluate the activity of the respective proteins. After normalization for protein concentration, an aliquot of the crude as well as the purified protein samples were diluted in TEGD buffer containing 1 mg/ml BSA and incubated in the presence of 3 nM [^{3}H]E2. The results in figure 3 demonstrate that the inclusion of the D domain had relatively little effect on activity, though it increased protein yield.

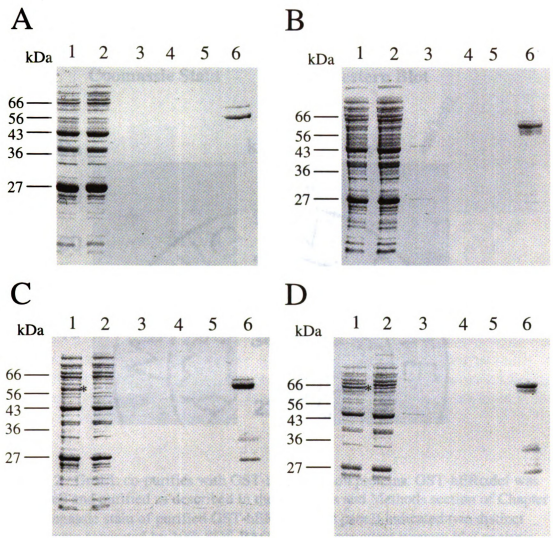

Figure 1. SDS-PAGE analysis of GST-rtERα purification. GST fusion proteins (A) GST-rtERα E, (B) GST-rtERα EF, (C) GST-rtERα DE, and (D) GST-rtERα DEF were expressed and purified as described in the Materials and Methods section. Various fractions were separated by 12% SDS-PAGE and visualized by coomassie blue staining. Lane 1: 20 μg of crude lysate, lane 2: 20 μg of flow through, lane 3: 10 μl of 10x concentrated wash I, lane 4: 10 μl of 10x concentrated wash II, lane 5: 10 μl of 10x concentrated wash III, lane 6: 3 μg of purified GST-rtERα protein. The asteriks identify the GST-rtERα fusion protein in the crude lysate.

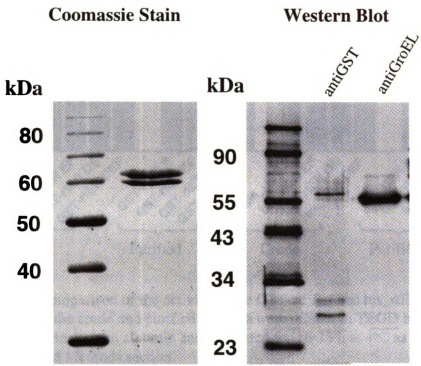

Figure 2. GroEL co-purifies with GST-ERdef fusion proteins. GST-hERαdef was extracted and purified as described in the Materials and Methods section of Chapter 3. Coomassie stain of purified GST-hERαdef (left panel) indicated two distinct bands were separated by 10% SDS-PAGE. After subsquent western blot analysis (right panel) the upper band was identified as GST-hERαdef, while the lower band was determined to be GroEL, a common contaminant of GST fusion protein preparations.

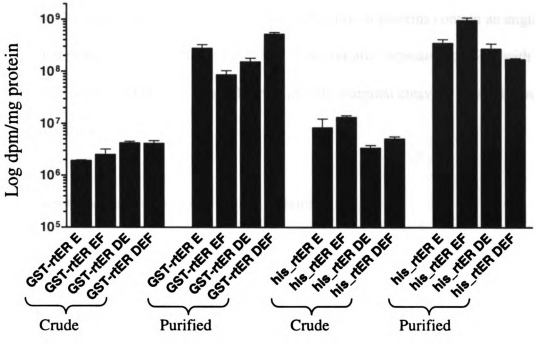

Figure 3. Comparison of the activity of the GST-rtERα and his_rtERα proteins. Aliquots of the crude and purified proteins were diluted in TEGD buffer containing 1 mg/ml bovine serum albumin and incubated for 16-18 h at 4°C as described in the Materials and Methods section.

One of the complications of using a GST fusion protein is the potential that the addition of the GST moiety may affect the activity of the protein and as a result it is generally removed by proteolytic cleavage. The GST-rtERα fusion proteins contain an engineered Precision protease site upstream of the rtER; however after repeated attempts with the GST-rtERαDEF and GST-hERαDEF proteins only marginal cleavage was observed (data not shown).

Expression and purification of his_rtER proteins

As an alternative to GST tagged proteins, the rtERα was also expressed as a 6x histidine tagged (his) protein and purified with nickel affinity chromatography. This approach proved superior to the GST-rtERα method in terms of overall protein expression, and purity, whereas similar levels of functional protein were observed. The results in figure 4 show that the his_rtERα E (30.0 kDa), his_rtERα EF (35.6 kDa), his_rtERα DE (34.4 kDa), and his_rtERα DEF (40.0 kDa) proteins migrated according to their predicted MWs with their purity ranging from 80-90%. However, there were significant contaminating bands in the his_rtERα EF preparation. Lower bands were also apparent in the his_rtERα DEF preparations, which most likely represent products of proteolytic cleavage, though they are not easily seen on the scanned gel presented in figure 4. Similar to that observed with the GST-rtERα proteins, the inclusion of the D domain dramatically increased protein yields with the expression of his_rtERα E, his_rtERα EF, his_rtERα DE, and his_rtERα DEF resulting in yields of 2.0, 2.0, 7.5, and 11 mg partially purified protein/L culture, respectively. A comparison of the crude

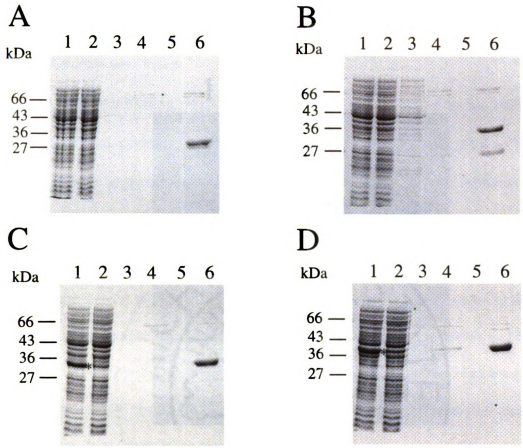

Figure 4. SDS-PAGE analysis of his-rtER purification. The his_rtER proteins (A) his_rtER E, (B) his_rtER EF, (C) his_rtER DE, and (D) his_rtER DEF were expressed and purified as described in the Materials and Methods section. Various fractions were separated by 12% SDS-PAGE and visualized by coomassie blue staining. Lane 1: 50 µg of crude lysate, lane 2: 50 µg of flow through, lane 3: 10 µl of 10x concentrated wash I, lane 4: 10 µl of 10x concentrated wash II, lane 5: 10 µl of 10x concentrated wash III, lane 6: 3 µg of purified his_rtER protein. The asteriks identifies the his_rtER protein in the crude lysate.

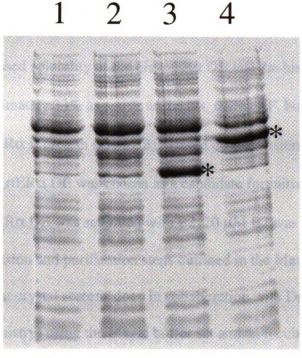

Figure 5. Influence of the D domain on the solubility of his_rtERα. Extraction of his_rtERα proteins from BL21 (DE3) cell pellets was performed as described in the Materials and Methods section. Equal amounts of crude protein extracts (30 µg) were separated by 12% SDS-PAGE and visualized by coomassie blue staining.

extracts from each of the four his_rtERα proteins shown in figure 5 clearly demonstrates the effect of the D domain on rtERα solubility. In figure 5, both the his_rtERα DE and his_rtERα DEF are visible in the crude extracts, while neither his_rtERα E nor his_rtERα EF were visible.

Receptor binding assays were used to assess the activity of the his_rtERα proteins and despite the increased solubility of the his_rtERα DE and the his_rtERα DEF both proteins were approximately 33% as active as the his_rtERα EF, but exhibited similar activity to the his_rtERα E protein (Figure 3). Based on expression, purity, and functionality, the his_rtERα DE was chosen as a candidate for initial crystallization trials. To ensure that the rtERα DE was saturated with E2, 20 μM E2 was added to all buffers throughout the expression and purification steps outlined in the Materials and Methods section. Two different crystal screens done in collaboration with Dr. Jim Geiger (MSU Departement of Chemistry) using the Magic buffer set and an incubation temperature of 16°C have failed to produce any crystals.

Discussion

Although the GST-rtERα and his_rtERα fusion proteins exhibit comparable ability to bind E2, there were marked differences in the purity and yield between both methods. The histidine tagged system was found to be superior to using GST as an affinity tag. Reasons for these differences may include the types of promoters that regulate the recombinant protein. The GST-rtERα fusion proteins are under the control of the ptac promoter, whereas the T7 promoter regulates his_rtERα protein expression. The GST moiety may inadvertently affect folding of the ER, thus leading to a larger proportion of insoluble products. Previous studies examining GST-rtERα CD and GST-rtERα EF constructs reported that nearly 100% of the expressed protein was insoluble (5).

The inclusion of the D domain to both GST and histidine tagged proteins significantly improved the yield by increasing the population of soluble protein. This was clearly evident in a comparison among the crude extracts from the his_rtERα proteins. Analysis of the amino acid sequence of the rtERα D domain (Chapter 2, figure 12 page 31) indicates that there are several charged hydrophilic residues that may influence solubility. The D domain is considered to be relatively flexible since it is thought to behave as a hinge, bridging the DNA binding and ligand binding domains together (11-13). This hydrophilic D domain may behave like a sequence tag that promotes ER solubility. In parallel studies in our laboratory a similar, albeit less dramatic effect, was observed with hERα.

Several nuclear receptors have been crystallized after expression and purification as recombinant fusion proteins in E. coli (14,15). The androgen receptor has been crystallized following expression and purification as GST fusions as well as histidine tagged proteins (16,17). Both moieties were proteolytically cleaved prior to concentration and crystallization (16,17). Since previous structural studies of the ER focused on the E domain, the his_rtERα E domain was initially selected for purification with the intent of x-ray crystallization. Subsequent analysis revealed that the his_rtERα E domain formed a precipitate during prolonged incubation at 4°C and/or when the protein was concentrated above 3 mg/ml. This solubility problem was not encountered with the his_rtERα DE construct and because of its increased expression and purity, the his_rtERα DE was chosen for initial crystallographic screens. In general one step purifications with the intent of crystallization are relative uncommon; however, the retinoic X receptor (RXR) DE has been successfully crystallized in this manner (18).

In this study, we describe a robust expression system that uses commercially available components and does not require specialized expression vectors (2). These data demonstrate that histidine tagged expression system is more suitable for the expression and purification of the rtERα than a GST based expression strategy. Overall protein yields and purity were best using the his_rtERα expression strategy. The hydrophilic D domain significantly improves solubility of the rtERα yielding 90-95% pure protein following a single step purification using metal affinity chromatography. Although this strategy has yet to produce any crystals, these screens are in their earlier stages and with a more thorough purification methodology may ultimate lead to the formation of crystals suitable for x-ray diffraction. In addition, an E2-affinity purification strategy, which has

been successfully used by several different groups to crystallize the hERα (19-21), could be used as an alternative to metal affinity chromatography.

References

1. Baneyx, F. (1999) *Curr Opin Biotechnol* **10**, 411-421.

2. Nygaard, F. B., and Harlow, K. W. (2001) *Protein Expr Purif* **21**, 500-509.

3. Le Drean, Y., Kern, L., Pakdel, F., and Valotaire, Y. (1995) *Mol. Cell. Endocrinol.* **109**, 27-35.

4. Pakdel, F., Le Gac, F., Le Goff, P., and Valotaire, Y. (1990) *Mol Cell Endocrinol* **71**, 195-204.

5. Pakdel, F., Petit, F., Anglade, I., Kah, O., Delaunay, F., Bailhache, T., Valotaire, Y. (1994) *Mol Cell Endocrinol* **104**, 81-93.

6. Greene, G. L., Nolan, C., Engler, J.P., Jensen, E.V. (1980) *Proc Natl Acad Sci U S A* **77**, 5115-5119.

7. Seielstad, D. A., Carlson, K.E., Katzenellenbogen, J.A., Kushner, P.J., Greene, G.L. (1995) *Molecular Endocrinology* **9**, 647-658.

8. Eiler, S., Gangloff, M., Duclaud, S., Moras, D., and Ruff, M. (2001) *Protein Expr Purif* **22**, 165-173.

9. Warnmark, A., Almlof, T., Leers, J., Gustafsson, J. A., and Treuter, E. (2001) *J Biol Chem* **276**, 23397-23404.

10. Rohman, M., and Harrison-Lavoie, K. J. (2000) *Protein Expr Purif* **20**, 45-47.
11. Giguere, V. (1999) *Endocr Rev* **20**, 689-725.

12. Zechel, C., Shen, X. Q., Chen, J. Y., Chen, Z. P., Chambon, P., and Gronemeyer, H. (1994) *Embo J* **13**, 1425-1433.

13. Zechel, C., Shen, X. Q., Chambon, P., and Gronemeyer, H. (1994) *Embo J* **13**, 1414-1424.

14. Watkins, R. E., Wisely, G. B., Moore, L. B., Collins, J. L., Lambert, M. H., Williams, S. P., Willson, T. M., Kliewer, S. A., and Redinbo, M. R. (2001) *Science* **292**, 2329-2333.

15. Bourguet, W., Ruff, M., Bonnier, D., Granger, F., Boeglin, M., Chambon, P., Moras, D., and Gronemeyer, H. (1995) *Protein Expr Purif* **6**, 604-608.

16. Matias, P. M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Joschko, S., Scholz, P., Wegg, A., Basler, S., Schafer, M., Egner, U., and Carrondo, M. A. (2000) *J Biol Chem* **275**, 26164-26171.

17. Sack, J. S., Kish, K. F., Wang, C., Attar, R. M., Kiefer, S. E., An, Y., Wu, G. Y., Scheffler, J. E., Salvati, M. E., Krystek, S. R., Jr., Weinmann, R., and Einspahr, H. M. (2001) *Proc Natl Acad Sci U S A* **98**, 4904-4909.

18. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375**, 377-382.

19. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**, 753-758.

20. Shiau, A. K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., Greene, G.L. (1998) *Cell* **95**, 927-937.

21. Goldstein, S. W., Bordner, J., Hoth, L. R., and Geoghegan, K. F. (2001) *Bioconjug Chem* **12**, 406-413.

CHAPTER 9

Conclusions and Future Directions

Conclusions

The data described above support our hypothesis that ERs among different vertebrate species exhibit differential binding affinity and in vitro gene expression responses to EEDs due to sequence divergence within their ligand binding domains. Although there were no obvious relationships between sequence identity and binding affinity and transactivation ability, (low sequence identity was not predictive of binding affinity of a compound), ERs with greater sequence identity exhibited similar relative binding affinities and transactivation abilities. The rtERα and mERβ, which have relatively low identity (60%) compared to hERα, exhibited the greatest differences among the ERs examined. Multiple sequence alignment identified amino acid residues that line the hormone binding pockets and differ among the vertebrate ERs examined. Taken together, this cross species comparison provides valuable insights into potentially important residues that may play critical roles in the interaction between structurally diverse ligands and the ER binding pocket. Although this study has identified several examples of EEDs exhibiting preferential binding affinity or ability to induce gene expression mediated by a specific vertebrate ER, the differences were relatively subtle and were rarely greater than an order of magnitude. This suggests the use of a single vertebrate ER to examine or screen for suspected EEDs is feasible, since the species-specific differences in binding and transactivation response of EEDs were relatively small.

Future Directions

1. Since, ERβ exhibits significantly greater affinity for EEDs, especially the phytoestrogens when compared to ERα. A comparison of the binding and transactivation profiles of EEDs for ERβs from representative vertebrate ERs would be an exciting continuation of the data presented above. ERβs exhibit significant amino acid sequence variability among vertebrate species. It would be interesting to examine whether vertebrate ERβs respond equally well to phytoestrogens and not just the mammalian ERβs.

2. During the course of the experiments described in chapters 3 & 4 we noted that the rtERα exhibits a temperature sensitive phenotype that was not seen in the other species examined. Although lowering the temperature significantly improved the function of the rtERα, an approximate 10-fold lower transactivation response compared to hERα was still observed. Using a reciprocal mutagenesis strategy, two amino acid residues (Met317, Ile496) were found to contribute not only to differences in binding and transactivation between hERα and rtERα but also to the reduced activity of the rtERα at elevated temperatures. The biological relevance of the rtERα exhibiting reduced function at elevated temperature is unclear, but may involve increased flexibility in the binding pocket of the rtERα compared to hERα that causes the rtERα to become unstable at elevated temperatures. In the absence of structural data the role these residues play influencing the position of E2 in the binding pocket is unclear. Similarly, the transactivation ability of another piscine ER, the Oreochromis aureus (OaER), also

exhibits reduced activity at temperatures above its normal physiological range. Interestingly, exchanging a 35 amino acid stretch (β region), between the OaER and the chicken ER (cER) partially rescued the thermal deficient transactivation of the OaER. Therefore, combining the reciprocal mutagenesis described above with the exchange of the β region between rtERα and hERα may result in complete phenotypic reversal between the two receptors.

3. A robust purification strategy was established to produce sufficient quantities of rtERα for further biophysical characterization. After comparing a series of different rtERα recombinant proteins his_rtERα DE was selected for initial crystallographic screens. Although the crystal screens have not produced any crystals, more extensive screens with different buffer conditions may enhance our success. Proteolytic removal of the 6x histidine tag combined with a more extensive purification strategy including ion exchange and/or size exclusion chromatography may also increase the probability of success. In crystallization studies of histidine tagged hERα E domain, only protein subjected to ion exchange gave crystals suitable for diffraction, while protein purified without ion exchange did not (3). Solving the crystal structure of the rtERα represents an important research challenge and hopefully the data presented here will contribute towards this goal.

References

1. Waller, C. L., Minor, D. L., and McKinney, J. D. (1995) *Environ Health Perspect* **103**(7-8), 702-707.

2. Tong, W., Perkins, R., Strelitz, R., Collantes, E.R., Keenan, S., Welsh, W.J., Branham, W.S., Sheehan, D.M. (1997) *Environ Health Perspect* **105**(10), 1116-1124.

3. Eiler, S., Gangloff, M., Duclaud, S., Moras, D., and Ruff, M. (2001) *Protein Expr Purif* **22**(2), 165-173.