MECHANISMS OF ARYL HYDROCARBON RECEPTOR AND ESTROGEN RECEPTOR ACTION IN BREAST CANCER CELLS

A Dissertation

by

JEONG EUN LEE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Toxicology

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Stephen H. Safe (Chair of Committee)	Kirby C. Donnelly (Member)
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ABSTRACT

Mechanisms of Aryl Hydrocarbon Receptor
and Estrogen Receptor Action
in Breast Cancer Cells. (December 2004)
Jeong Eun Lee, B.S., Seoul Women's University;
M.S., Seoul Women's University
Chair of Advisory Committee: Dr. Stephen Safe

In MCF7 and T47D cells cotreated with 1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) plus 0.1-10 μM 3',4'-dimethoxy flavone (DMF), there was a concentration-dependent decrease in the TCDD-induced ethoxyresorufin O-deethylase (EROD) activity. Gel mobility shift assays showed that 3',4'-DMF inhibited TCDD-induced aryl hydrocarbon receptor (AhR) transformation in rat liver cytosol and blocked TCDD-induced formation of the nuclear AhR complex in MCF7 and T47D cells. The antiestrogenic activity of TCDD in estrogen-induced transactivation assays in MCF7 cells was reversed by 3',4'-DMF, confirming the AhR antagonist activity of this compound in breast cancer cells.

Cotreatment of T47D and MCF7 cells with TCDD and 10 μM resveratrol inhibited induction of CYP1A1 mRNA and EROD activity. Resveratrol did not inhibit TCDD-induced AhR transformation and reporter gene activity. Actinomycin D chase experiments in T47D cells showed that the mechanism of inhibition of CYP1A1 mRNA and EROD activity is due to an increased rate of CYP1A1 mRNA degradation,

suggesting that resveratrol inhibits CYP1A1 via an AhR-independent post-transcriptional pathway.

Vitamin D receptor-interacting protein 150 (DRIP150) coactivated estrogen receptor α (ERα)-mediated transactivation and the response was AF2-dependent in ZR75 breast cancer cells. C-and N-terminal NR-boxes (amino acids 1186-1182 and 73-69, respectively) were not necessary for coactivation of ERα. Analysis of DRIP150 deletion mutants identified a 23 amino acid sequence (811-789) required for coactivation. The 23 amino acid contained two regions at amino acids 789-794 and 795-804 which resembled α-helical motifs identified in Lanuguinosa lipase/histamine N-methyl transferase and hepatocyte nuclear factor 1, respectively. A squelching assay using specific point mutations within each α-helix showed that the NIFSEVRVYN (795-804) region was the critical sequence required for the coactivator activity of DRIP150.

DEDICATION

To my parents, Chang Bo Lee and Jung Ok Lee

My sister and brother-in-law, Jeung A Lee and Eung Ho Lee

For their love, support, and patience.

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CHAPTER I

INTRODUCTION

Identification of AhR

Individuals are constantly exposed to a variety of low molecular weight compounds including secondary plant metabolites, mycotoxins, venoms, pharmaceuticals, and the by-products of industrialization. For several decades scientists have been aware that adaptive mechanisms exist to minimize toxicity from these ubiquitous dietary and environmental compounds. A metabolic response to polycyclic aromatic compounds (PAHs) combustion by-products was first described in the late 1950s. In these early rodent experiments, the administration of benz[a]anthracene, benzo[a]pyrene or 3-methylcholanthrene (MC) led to the induction of a number of liver microsomal enzyme activities collectively referred to as arylhydrocarbon hydroxylase (AHH) (1). This induced metabolism met the criterion of an adaptive response in that the upregulated enzymes were able to oxidize the same PAH-inducing agents upon short-term re-exposure. Similar adaptive responses were also observed for other classes of structurally unrelated xenobiotics such as phenobarbital, other barbiturates, drugs and various pesticides. Initial exposures to these compounds led to increased expression of microsomal and soluble enzymes with metabolic activity toward the inducing agent resulting in a decreased pharmacological response unless a metabolite was the active agent (2).

This dissertation follows the style of the *Journal of Biological Chemistry.*

Classical murine genetics provided initial insights into the regulation of AHH activity. First, it was observed that the inducibility of AHH activity varied significantly among inbred mouse strains, with C57 strains being highly responsive to PAHs, whereas the DBA and AKR strains were described as nonresponsive (3). Crosses and back-crosses of these strains indicated that multiple alleles at a single locus controlled inducibility of AHH. This locus initially became known as Ah, for aryl hydrocarbon responsiveness (4,5). Although the terms responsive and nonresponsive are still widely used, their application should be limited to induction by PAHs, because halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) soon were found to be orders of magnitude more potent than PAHs and were capable of eliciting AHH induction in nonresponsive strains (6). These results demonstrated that nonresponsive strains were actually less responsive, requiring 10-to100-fold higher doses of TCDD to attain the same level of enzyme induction observed in responsive strains.

Data from a number of laboratories led to the suggestion that a receptor existed for this large class of chemicals and that C57 mice harbored a receptor with a greater affinity for ligand than the corresponding receptor in DBA mice (7). The existence of this Ah receptor (AhR) was confirmed using radiolabeled TCDD congeners to demonstrate the existence of low-capacity, high-affinity binding sites in mouse hepatic cytosol (8). As predicted, the binding affinity for TCDD for receptors differed between mouse strains, with receptors from the responsive and nonresponsive strains displaying equilibrium dissociation constants of 6 and 37 pM, respectively (9). The segregation of these alleles, as well as structure-activity studies performed with TCDD and related compounds, confirmed the existence of the AhR and its role in regulating the induction

of AHH. The idea that the *Ah* locus encoded the AhR resulted in the recent renaming of this locus to *Ahr* by the Mouse Genome Nomenclature Committee (10).

The AhR is highly polymorphic, particularly when compared with other nuclear receptors. This polymorphism extends beyond the classical responsive and nonresponsive phenotypes described above to include significant differences in receptor primary structure. For example, marked differences in AhR molecular weight have been revealed with the use of [^{125}I]-photoaffinity ligands and antibodies (11,12). Three different *ahr* alleles, denoted with a “b” superscript from the prototype C57BL strain, have been identified that encode high-affinity receptors in responsive strains. The allele found in C57 strains, *Ahr*$^{b\text{-}1}$, encodes a 95-KDa receptor with high affinity for TCDD, whereas a 104-KDa high-affinity allele, *Ahr*$^{b\text{-}2}$, is found in most other commonly used laboratory strains such as C3H/He and BALB/c (13). Several wild-mouse strains, including *Mus spretus, caroli*,and *molossinus*, harbor a third high-affinity allele, *Ahr*$^{b\text{-}3}$, encoding a 105-KDa receptor protein (14). At present, only a single allele has been identified that encodes for the low-affinity receptor in nonresponsive strains (*Ahr*^{d}) (15). This allele is denoted with a “d” superscript, from the prototype DBA strain, and encodes a receptor protein of 104 KDa (14). The structural and functional variability of the AhR is also evident across species. Photoaffinity labeling of hepatic cytosol indicates that the AhR can vary in molecular weight by almost 30 kilodaltons, e.g. C57 mouse, 95; chicken,101; guinea pig, 103; rabbit, 104; rat,106; human,106; monkey,113; and hamster, 124 (12). Recent cloning studies have demonstrated that this difference in molecular weight is primarily due to differences in the position of the AhR’s translational termination codon, rather than differential splicing or posttranslational modifications (16-19).

Structure/function of AhR and Arnt

The basic-helix-loop-helix (bHLH) motif has been described in a wide variety of transcription factors such as the mammalian proteins Myc, Max, MyoD, and E2A, and the *Drosophila* proteins Achaete-scute and Daughterless (20,21) that function as sequence-specific transcriptional regulators. This motif has been demonstrated to harbor subdomains that play roles in both DNA binding (basic region) and protein dimerization (HLH) (20,22,23). A feature of many bHLH proteins is the presence of a secondary dimerization surface adjacent to the HLH domain. One well characterized example of such a secondary dimerization domain is the lucine zipper, and bHLH proteins containing this motif are called bHLH-ZIP proteins (21). Myogenic determination protein MyoD and its relatives Myogenin, Myf-5 and MRF4 are among the most widely studied members of the bHLH proteins and illustrate many of the general features of these proteins (24,25). The myogenic bHLH proteins were identified based on their ability to activate muscle-specific genes and induce muscle cell differentiation in nonmyogenic cells. These factors autoregulate their own expression and cross-regulate the expression of the other family members. Studies on the regulation of skeletal muscle development provide evidence for distinct roles for each of the myogenic factors in both determination and differentiation of muscle cell phenotype (26-28). All four myogenic factors form heterodimers with the E12 and E47proteins, which are alternately spliced products of the E2A gene, to generate functional DNA-binding complexes (20,24,29). Regulation of this system is maintained under different physiologic conditions not only by the complement of dimeric partners that are expressed, but also by restricting the heterodimeric pairs that may form. Key regulators of partner availability are two dominant-negative inhibitory proteins, Id1 and Id2 (29,30).

These proteins have been shown to interact with E12 and E47, as well as with MyoD, forming nonfunctional complexes devoid of DNA-binding ability.

Cloning of the AhR and Ah receptor nuclear translocator (Arnt) genes allowed amino acid sequence alignments, which revealed that these two proteins are similar in primary amino acid sequence to the *Drosophila* proteins Sim and Per (29-36). The homologous domain present in all four proteins has been termed the PAS domain, for Per-Arnt-Sim. In addition to the PAS motif, the AhR , Arnt, and Sim also have adjacent bHLH domains (Fig. 1). The AhR and Arnt are bHLH-PAS proteins. Sim is a bHLH-PAS protein involved in the specification of cell fate during midline cell differentiation in *Drosophila* (37,38). Per, known to be involved in the maintenance of circadian rhythms, is the most unusual member of this family in that it does not contain a bHLH region and may function as a dominant –negative inhibitor in a manner similar to the Id proteins of the MyoD system (30). Very recently, a number of new members of the bHLH-PAS superfamily have emerged from cloning studies. The hypoxia-inducible factor 1α (HIF-1α), a regulator of cellular response to hypoxic stress, was purified and cloned from hepatoma cells and two additional bHLH-PAS members are the products of the *similar* and *trachealess* genes of *Drosophila* (39,40). Together these cloning and sequencing studies suggest that a superfamily of PAS proteins exists in a wide variety of cell types and organisms.

Fundamental questions remain concerning the endogenous function of the AhR and its role in the toxicity of TCDD. The use of gene targeting technology to inactivate murine genes in vivo (knockout mice) has been a powerful technique to elucidate protein function, confirming predicted actions in some cases while uncovering unexpected roles in others. The *Ahr* gene is an ideal candidate for targeted

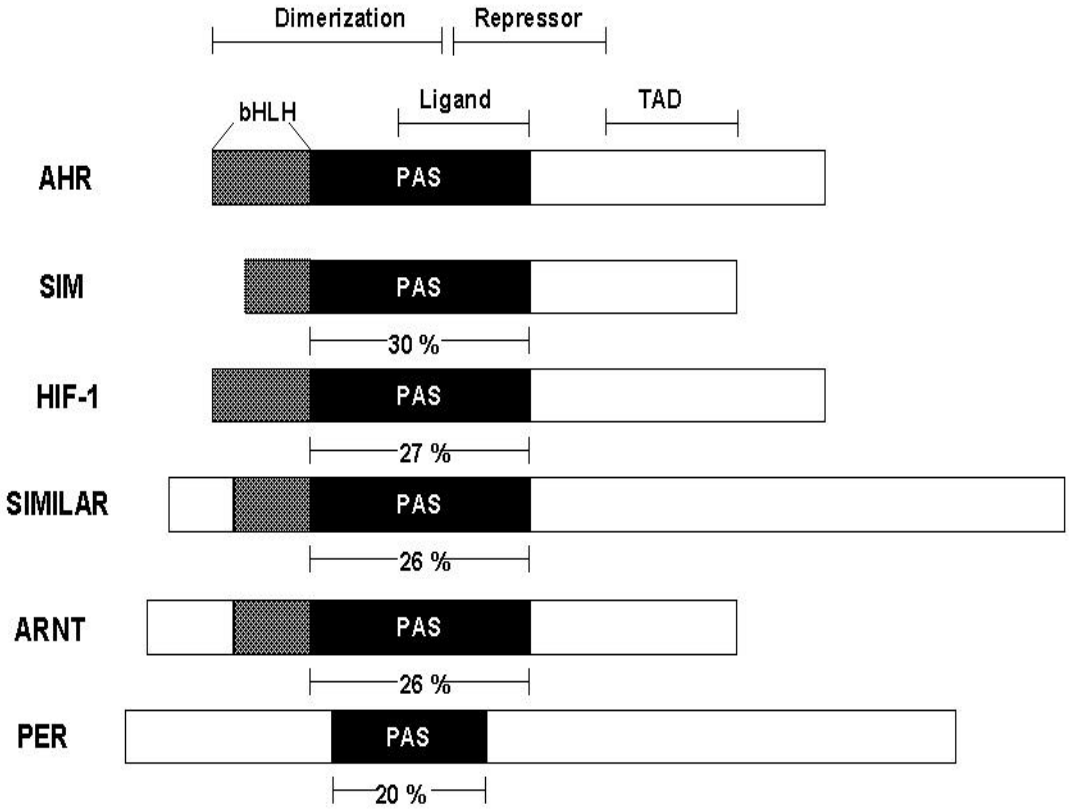


FIG. 1. **Schematic representation of the bHLH-PAS family proteins.** The stippled areas represent the bHLH region and the black areas the PAS domain. For the AhR, the region marked Dimerization indicates bHLH and PAS sequences required for AhR-Arnt dimerization and therefore also for DNA binding. The region marked Ligand indicates the ligand-binding domain as mapped by photoaffinity labeling of deletion constructs, and the region marked TAD indicates the transactivation domain. For the other bHLH-PAS family members, percent amino acid identity to the AhR within the PAS region is indicated beneath each protein. Per does not contain a bHLH domain.

inactivation; *Ahr* null mice might demonstrate an unknown AhR function (endogenous pathway) and provide a valuable model system for investigation of TCDD-induced toxicity. *Ahr* null mice have been generated independently by two groups, yielding very different phenotypes (41,42).

Schmidt et al has used gene targeting to delete exon2, which encodes the bHLH DNA-binding and dimerization domain, generating an *Ahr* null mouse line (41). RT-PCR analysis detects the presence of a full-length alternatively spliced *Ahr* transcript, lacking exon2, produced from the targeted allele. This splicing event generates a frame-shift, and detailed Western blot, as well as functional assays, detect no AhR protein in this model system. The $Ahr^{\triangle 2/\triangle 2}$ animals are viable and fertile; however, they exhibit a spectrum of hepatic defects suggesting that the AhR may play a previously unrecognized role in liver growth and development. $Ahr^{\triangle 2/\triangle 2}$ mice appear normal at birth but display slowed growth for the first few weeks of life. At 1 week of age, these animals show a dramatic yet transient liver phenotype that includes decreased liver weight, fatty metamorphosis, and increased residual extramedullary hematopoiesis. Detailed analysis of the decrease in liver weight has shown that this aspect of the $Ahr^{\triangle 2/\triangle 2}$ phenotype is present at all ages examined so far, from birth through 6 weeks. The fatty change of the liver, however, develops after birth and resolves entirely by 3 weeks of age. The residual extramedullary hematopoiesis also resolves by this age. Older $Ahr^{\triangle 2/\triangle 2}$ mice (beyond 3 weeks of age) begin to develop mild portal hypercellularity with thickening and fibrosis, and approximately 50 % of animals have enlarged spleens by 6 weeks. Although the underlying basis for this phenotype is unknown and will be the subject of much future study, it may represent a hepatic developmental delay. The phenotype may indicate a role for the AhR in liver growth and

maturation to a functionally metabolic organ. In addition to providing other functional roles for the AhR, these mice will serve as valuable tools to distinguish between receptor-mediated from nonreceptor-mediated effects of various AhR agonists.

Ahr mice that display a quite different phenotype from $Ahr^{\triangle 2/\triangle 2}$ were first generated by Fermandez-Salguero at al (42). This group targeted their inactivating mutations to the first exon of the *Ahr*, deleting the initiation methionine and a portion of the basic region. In $Ahr^{\triangle 1/\triangle 1}$ mice P4501A1 is not induced in response to TCDD. The mice display a 50% neonatal mortality rate, with inflammation of several major organ systems. Surviving $Ahr^{\triangle 1/\triangle 1}$ mice have decreased liver weights and portal fibrosis similar to that seen in the $Ahr^{\triangle 2/\triangle 2}$ mice; however, both phenotypes appear to be more severe in the $Ahr^{\triangle 1/\triangle 1}$ mice. Additionally, the $Ahr^{\triangle 1/\triangle 1}$ animals have a severely depressed immune system, with an 80% decrease in total splenic lymphoid cells at 2 weeks of age that gradually resolves over time. This indicates a role for the AhR in neonatal lethality and immune function. However, there is no evidence of neonatal lethality or immune cell depletion in $Ahr^{\triangle 2/\triangle 2}$ mice. The reasons underlying the phenotypic difference between the two *Ahr* null mouse lines remain unclear.

AhR-mediated toxicities

Genetic, biochemical, and molecular biology studies have revealed that the AhR mediates the toxic and biological effects of environmentally persistent TCDD and related compounds. The AhR was first described and studied based on its ability to bind and mediate the toxic and biological effects of PAHs, HAHs, and related compounds. The HAHs include a wide group of compounds such as polychlorinated dibenzo-p-dioxin (PCDD), dibenzofurans (PCDFs), biphenyls (PCBs), diphenyl ethers, naphthalenes and others (Fig. 2). There are 75 PCDD, 135 PCDF, 209 PCB congeners

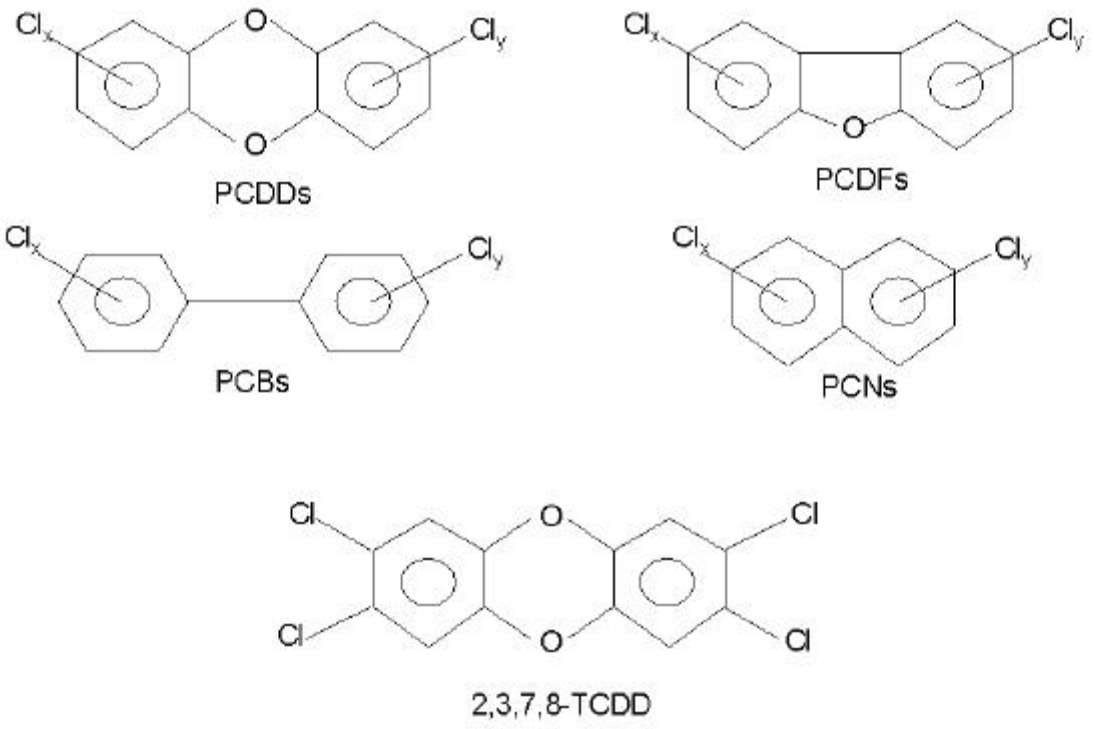


FIG. 2. **Structures of several classes of HAHs.**

and hundreds of polychlorinated naphthalenes, azo- and azoxy-benzenes, terphenyls, quarterphenyls and biphenylenes. 2,3,7,8-TCDD or dioxin is the prototypical and most toxic member of this class of compounds. The term “dioxin” is used to indicate either TCDD specifically, or PCDD family in general. Biologically, TCDD is the most potent PCDD (43).

The HAHs elicit a diverse spectrum of sex, strain, age, species, and tissue specific responses, which include body weight loss, thymic atrophy, immunotoxicity, hepatotoxicity and porphyria, chloracne and related dermal lesions, tissue specific hypo- and hyperplastic responses, carcinogenesis, teratogenicity, reproductive toxicity, and numerous biological responses such as the induction of phase I and phase II drug-metabolizing enzymes(44). The role of the AhR in mediating the pleiotropic responses elicited by HAHs can be inferred from two major lines of evidence. Firstly, for several of these responses, including dermal toxicity, immunotoxicity, porphyria, thymic atrophy, body weight loss, acute lethality (LD_{50} values), and teratogenicity, the potencies of several PCDD, PCDF, and PCB congeners are structure-dependent. Moreover, the structure-toxicity relationships for halogenated AhRs are similar to the structure-induction AHH relationships and the most active congeners exhibit relatively high AhR binding affinities and are approximate isostereomers of 2,3,7,8,-TCDD. Secondly, pharmacogenetic studies with genetically inbred mice and their backcross provided the initial evidence which supported the role of the AhR in the induction of AHH by TCDD and 3-methylcholanthrene (MC). Subsequent research with both Ah responsive (Ah^b allele) and nonresponsive (Ah^d allele) strains of mice demonstrated that several toxic effects elicited by TCDD, including hepatotoxicity, immunotoxicity, porphyria, body weight loss, and teratogenicity, segregate with the Ah locus. Allelic differences in

the murine Ah locus have been associated with carcinogenicity at distal and proximal sites, neonatal toxicity, immunotoxicity, susceptibility to atherosclerosis, bone marrow toxicity, and numerous other toxic effects. Some of these effects are related to differential induction of cytochrome P-4501A1 and the subsequent modulated metabolism of toxins/carcinogens (45).

A surprising number of deleterious biological responses in human have been shown to result from TCDD exposure and these include epithelial hyperplasia, chloracne, induction of drug-metabolizing enzymes, altered estrogen receptor (ER) signaling, porphyria, deregulated lipid metabolism, decreased serum thyroxine, wasting, metabolism of arachidonic acid to biologically active products, vitamin A depletion, cardiac dysfunction, utilization of brown adipose tissue, teratogenesis/embryotoxicity, inhibition of gluconeogenesis, immunosuppression, lipid peroxidation, epidermal growth factor receptor (EGFR) down-regulation, persistent thyroid hormone receptor activation, and tumor promotion(46).

TCDD has a half-life of about 10-15 days in mice (47,48), 12-31 days in rats and 5-10 years in humans. TCDD exhibits wide inter-and intra-species differences in LD_{50} values (Table I) that range from 0.6 μg/kg in male guinea pigs (49) to 5500 μg/kg in hamsters(50).

TCDD reduces fertility, litter size, uterine weights, alters ovarian function, increases incidence of spontaneous abortions and disrupts normal estrus cycling in several mammalian species. Studies have shown that TCDD delays vaginal opening and induces cleft phallus/clitoris in female rats. In several cases of clefting, the animals were also hypospadic. TCDD inhibits several estrogen-induced responses in the rodent uterus and modulates several endpoints regulated by estrogen such as uterine

TABLE I
Acute lethality of TCDD to various species and strains

Species/strain/sex	Route	LD_{50} (μg/kg)	Time of death (days post exposure)	Follow-up (days)	Body weight loss
Guinea pig	oral	2	>5	30	50
Mink	oral	4.2	7-17	28	31
Rhesus monkey	oral	~70	14-34	42-47	13-38
Rat/S-D/Male	ip	60	NP	20	NP
Rat/S-D/Female		25			
Mouse/C57BL/6	oral	182	24	30	25
Mouse/DBA/2		2570	21		33
Mouse/B6D2F1		296	25		34
Rabbit	oral	115	6-39	NP	NP
	ip	275	12-22	22	NP
	dermal	~50	7-10	10-20	11
Golden-syrian hamster (male)	oral	5051	9-43	55	NP

NP-not provided; (51)

peroxidase activity, EGFR levels, progesterone receptor (PR) levels and *c-fos* oncogene expression(52). Reproductive toxicity of TCDD in humans has been difficult to assess but is believed to cause an increase in spontaneous abortions and birth defects such as spina bifida and cleft palate (53).

In several species such as rhesus monkeys, rats, mice, guinea pigs and chickens, treatment with TCDD causes various alterations in testicular morphology including loss of germ cells, appearance of degenerating spermatocytes and mature spermatozoa within the lumen of the seminiferous tubules and a reduction in the number of tubules containing mature spermatozoa. The ED_{50} for this response in rats is 15 μg/kg and is accompanied by signs of overt toxicity, such as wasting syndrome, indicating that these are high-dose effects (54).

Epidemiological studies show an association between dioxin exposure and decreased serum testosterone levels and increased serum follicle-stimulating hormone (FSH) and lutenizing hormones (LH) in male workers exposed to TCDD during the manufacturing of 2,4,5-trichlorophenol (55)

Developmental toxicity caused by TCDD-like congeners has been extensively documented in fish, birds and mammals and commonly includes decreased growth and prenatal mortality (56).In fertilized eggs from lake trout, the LD_{50} was 65 pg of TCDD (waterborne)/g egg. Injection of TCDD-like congeners in chicken eggs resulted in liver lesions, edema, thymic hypoplasia, beak deformations and cardiac malformation; however, none of these symptoms were observed in turkey eggs indicating that TCDD-induced effects in bird embryos are highly species dependent (57). The embryo or fetus is more susceptible to TCDD than adults of the same species. The LD_{50} for TCDD in rainbow trout sac fry is 25 times lower than that in juvenile rainbow trout; 100-200 times less in the chick embryo compared to adult chickens and 64-280 times lower in hamster fetus compared to adult hamsters (51).

In mammals, especially mice, TCDD exposure causes several structural malformations. These include cleft palate and hydronephrosis (58). Epidemiological studies have reported that the offspring of women exposed to HAH mixtures developed ectodermal dysplasia characterized by hyperpigmentation of the skin, mucous membranes, fingernails, toenails, presence of erupted teeth in neonates and hypersecretion of the meibomian gland. In addition, in utero exposure to these mixture also resulted in neurobehavioral abnormalities and delays in developmental milestones (59).

TCDD disrupts the endocrine system by altering responses that are involved in

homeostatic control at the cellular, tissue, organ and organism levels. The anterior lobe ot the pituitary gland is a target for TCDD and exhibits both exaggerated and suppressed responses to its regulatory hormones following TCDD exposure. A 50 μg/kg dose of TCDD decreased prolactin concentration within 4 h in male Sprague-Dawley (S-D) rats (60). TCDD enhanced thyroid stimulating hormone directly or via the hypothalamic releasing hormone (51).

TCDD does not compete with glucocorticoids in binding to their intracellular receptors but it diminishes the binding capacity of hepatic glucocorticoid receptors, in a cell- and tissue-dependent manner, in the rat and mouse (61). Adrenalectomy increased mortality of male S-D rats exposed to TCDD (62). Studies have shown that adrenalectomy sensitizes rats to hepatic monooxygenase induction as well as glucocorticoid receptor downregulation by TCDD (51).

TCDD decreases serum insulin levels in rats (63) and studies with isolated pancreatic membranes from TCDD-treated guinea-pigs showed increased protein-tyrosine kinase activities(64). A high nonlethal dose of TCDD (25 μg/kg) decreased plasma glucagon whereas a lethal dose (125 μg/kg) significantly increased glucagon levels in S-D rats (62).

TCDD reduces serum T_4 concentrations rapidly in rats but has a variable response on T_3 (65). TCDD is believed to exert its effects on serum thyroid hormones in rats by accelerated clearance of T_4 (66) through selectively enhanced biliary excretion (67). Studies have suggested that hydroxylated metabolites of TCDD may compete with T_4 for binding to transthyretin leading to TCDD-induced decline in serum T_4 levels. TCDD also modulates concentrations of thyroid-hormone receptors. TCDD exposure resulted in an increase in mRNA for *c-erb-A* oncogene in livers of C57BL/6 mice but not

in DBA/2 mice implying elevated concentrations of nuclear T_3 receptors by TCDD in the former strain (68).

TCDD dramatically decreases circulating melatonin levels but not pineal melatonin content (51). Studies suggest that decreased serum melatonin was possibly due to accelerated peripheral clearance following TCDD exposure (69). TCDD-induced changes in this important day/night signaling substance may contribute to biological effects such as shifts in corticosterone or feeding rhythms (70).

The toxic effects of HAHs are mediated through alterations in normal homeostatic processes that are regulated through interactions of growth factors, steroid hormones, and enzymes involved in the synthesis and degradation of these factors. TCDD can alter the levels of both growth factors and their receptors (56).

In vivo studies, in the hepatic plasma membranes of rats and guinea pigs, have shown that TCDD increases protein kinase activities such as protein kinase C (PKC) (71) and several tyrosine kinases (72). Induction of PKC by TCDD has been demonstrated in the rat testis(73), rat thymus(68), and in primary cultures of rat hepatocytes and thymocytes (74). TCDD also stimulates *c-ras* protooncogene product in hepatic plasma membranes. Studies have also reported that TCDD activates phospholipase C (75).

HAHs induce phase I and phase II drug-metabolizing enzymes. These include a broad spectrum of P450-dependent monooxygenase enzyme activities including several PAH hydroxylases, steroid hydroxylases, *O*-dealkylases, several haloaromatic hydroxylases, N-dealkylases, and barbituate hydroxylases. Phase II enzymes include epoxide hydrolase, glucuronosyl transferase, glutathione-S-transferase and reductase (52). TCDD can induce the level and activity of a number of enzymes involved in

metabolism, particularly of endogenous substrates.

TCDD is classified as a probable human carcinogen by the EPA and as a possible human carcinogen by IARC (76). TCDD is a trans-species (rat, mouse and hamster), trans-strain (Sprague-Dawley and Osborne-Mendel rats; B6C3F1, Swiss-Webster and B6C mice), trans-sex, multisite complete carcinogen. TCDD is a potent carcinogen in laboratory animals with tumors observed at doses as low as 0.001 μg/kg body weight per day (77,78). In rats, TCDD induced neoplasms in the lung, oral, and nasal activities, thyroid and adrenal glands and liver (79). In mice, TCDD-induced neoplasms were seen in the liver, subcutaneous tissue, thyroid gland, lung, and lymphopoietic tissue (80). In hamsters, TCDD produced squamous cell carcinoma of the facial skin (81). Epidemiological data from occupationally exposed workers show that TCDD exposure is associated with several cancers in humans: respiratory, lung, thyroid gland, connective and soft tissue sarcoma, hematopoietic system, liver and all cancers (82-86).

Carcinogenic effects of TCDD are produced via a receptor-mediated, nongenotoxic, epigenetic mechanism. In two-stage liver and skin models, TCDD exhibits considerable promotional activity (87). TCDD is a promoter of liver tumors following initiation with a single 10 mg/kg dose of diethylnitrosamine (DEN), with an increase in the number of foci and the production of well-differentiated hepatocellular carcinomas. The PCB congeners that most actively promote hepatic tumors (3,3',4,4'-tetraCB and 2,3,4,4',5-penta CB) are also inducers of hepatic CYP1A1-dependent monooxygenases. In addition to promotional activity, particularly in the liver, PCBs can alter the metabolism of other carcinogens also, thereby increasing cancer incidence.

Mechanisms of AhR action

The AhR is primarily a cytosolic protein complexed with two 90-kDa heat shock proteins (Hsp90) and lower molecular weight chaperons and this complex does not bind DNA (88,89). In a manner similar to steroid hormone receptors, Hsp90-AhR interactions are destabilized by ligand binding, a process referred to as transformation (90). The ligand-activated AhR heterodimerizes with the bHLH protein Arnt forming a dimeric complex that binds to xenobiotic regulatory elements (XREs) in target gene promoters such as CYP1A1. Induction of CYP1A1 involves the classical activation cascade of the AhR, e. g. binding of the ligand to the AhR, heterodimerization with Arnt protein, formation of a complex with XRE and subsequent gene activation (Fig. 3).

Some xenobiotics activate CYP1A1 gene expression in spite of their inability to compete with TCDD for AhR binding and ligand-independent induction includes stress conditions such as hyperoxia and hydrodynamic shearing and enhanced AhR transcription during the differentiation of monocytes and keratinocytes (91,92).

CYP1A1 inducers that do not bind the AhR have been categorized into structurally-related chemical families. For example benzimidine family members which enhance CYP1A1 include omeprazole (OME), which inhibits H^+/K^+-ATPase, and is currently used in the treatment of gastric ulcers. The fungicide thiabendazole (TBZ) .also induces CYP1A1. The second structural groups are the carotenoids (precursors of retinoids) canthaxanthin and ß-apo-8'-carotenal. Finally two other structurally unrelated compounds also induce CYP1A1; carbaryl an important insecticide which belongs to the carbamate family and primaquine a drug which is of extreme importance in malaria chemotherapy. These results suggest that other signaling pathways may be involved in activation of CYP1A1. Retinoids are capable of inducing CYP1A1 through

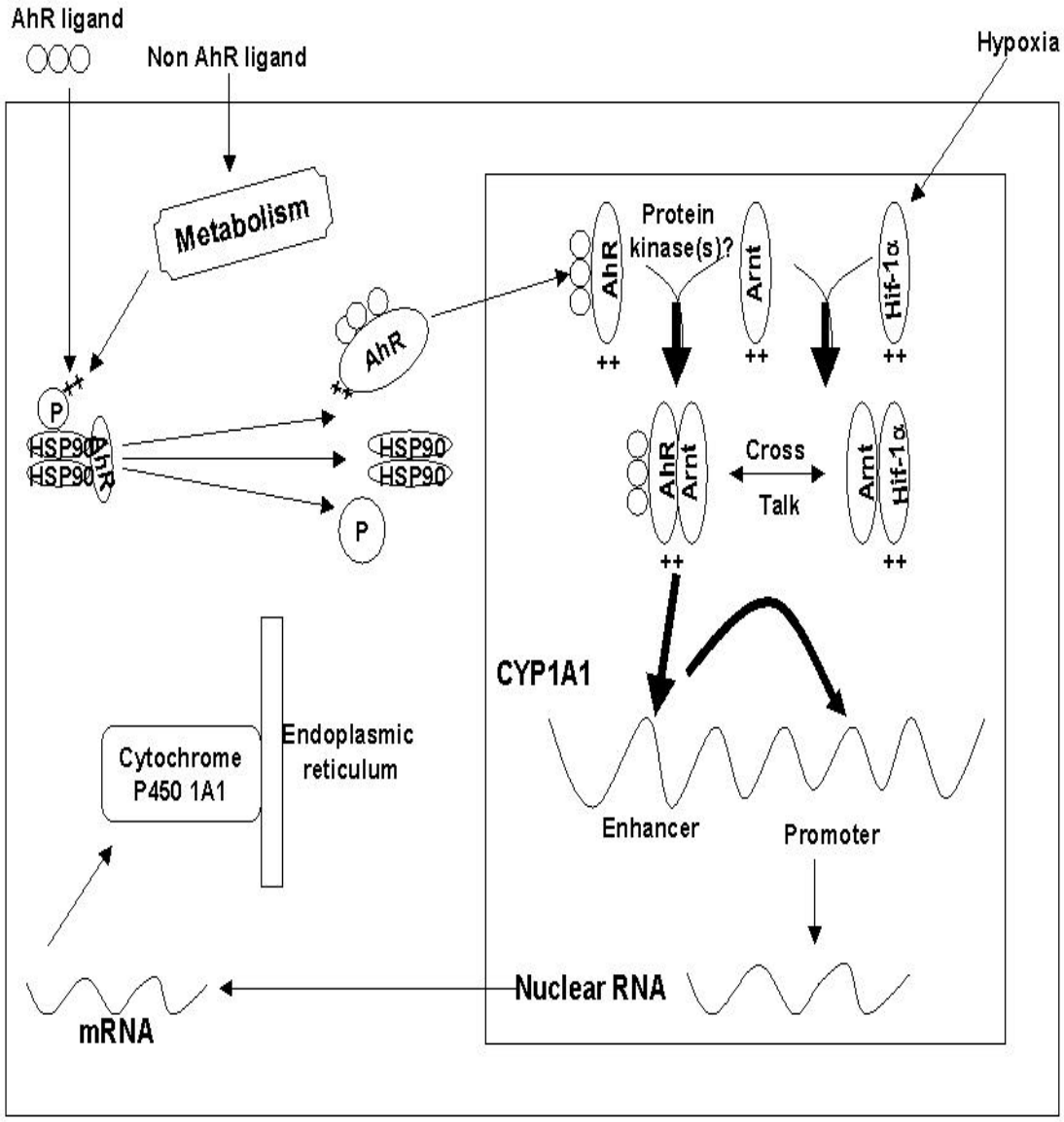


FIG. 3. **General CYP1A1 induction model also known as the AhR signaling transduction pathway.**

retinoic acid responsive element (RARE). Carotenoids, which are precursors of retinoids, and thereby structurally related may activate CYP1A1 through the retinoic acid receptor (RAR) signaling pathway. Crosstalk between the AhR and RAR signaling pathways may be related to modulation of RAR-dependent pathways. Tyrosine kinase activation may also play a role in ligand-independent activation of CYP1A1 however the mechanism of this response is unknown. For omeprazole, carbaryl and primaquine, CYP1A1 induction by these chemicals is inhibited by a PKC inhibitor, staurosporine, suggesting that these chemicals could activate PKC. AhR and Arnt are both phosphoproteins and phosphorylation of the AhR seems to occur predominantly on its carboxyterminal end. Furthermore, AhR and Arnt are both phosphorylated on threonine residues, suggesting that a serine/threonine kinase may be directly or indirectly involved in regulation of AhR/Arnt functions. Overall, little is known about PKC-dependent regulation of AhR or Arnt activities. However, recent studies showed that PKC-dependent phosphorylation of both AhR and Arnt is required for the classical AhR signaling pathway in some tissues/cells suggesting that other types of CYP1A1 inducers that modulate phosphorylation may facilitate assembly of a fully functional transcription complex (93).

Several studies have reported interactions of AhR and ER signaling pathways in a number of tissues/cells and this includes the following pathways: 1) the AhR may suppress ER-induced gene expression by interactions of the AhR with critical regions of 17β-estradiol (E2)-responsive gene promoters, 2) the AhR induces downregulation of ER levels, 3) the AhR increases the metabolism of E2, and 4) activation of AhR may release Hsps that inhibit ER signaling while squelching ER-dependent coactivators.

The presence of inhibitory DREs (iDREs) which interact with the AhR may disrupt or prevent ER-mediated transcription and these have been identified in cathepsin D, pS2, Hsp27, and *c-fos* gene promoters (94-97). Induction of the cathepsin D gene by E2 requires the binding of both ER and Sp1 to promoter elements, and activated AhR binds to a DRE located between the ERE and Sp1 motif, preventing ER/Sp1-DNA binding (Fig. 4). Identification of a functional iDRE in the cathepsin D gene promoter suggested that this same motif may play an important role in inhibitory AhR-ER crosstalk and functional iDREs have now been characterized in other E2-responsive genes. Zacharewski and coworkers first showed that TCDD inhibited E2-induced pS2 gene expression in breast cancer cells and promoter analysis identified a functional iDRE (GCGTG at –521 to –517) over 100 bases upstream from an imperfect palindromic ERE (-405 to –393) that binds ERα and is required for E2-responsiveness (96). The mechanism of inhibitory AhR-ER crosstalk associated with the pS2 gene promoter is due to competitive AhR/Arnt-AP1 binding to overlapping response elements and subsequent modulation of AP1-ER interactions (Fig. 4). Similar interference with DNA-bound transcription factors has been observed in regulation of other gene promoters (98). In the Hsp27 promoter, the functional iDRE is located near the transcription start site and may act by interfering with the general transcription machinery (Fig.4). Inhibitory AHR-ER crosstalk for the *c-fos* gene is associated with quenching or masking of DNA binding by competing transcription factors since the results from gel mobility shift and DNA footprinting assays indicate that the AhR/Arnt and ER/Sp1 compete for binding at the overlapping GC-rich/iDRE motif (Fig.4). In addition to cathepsin D, pS2, Hsp27 and *c-fos* gene promoters, E2F1, adenosine deaminase, insulin-like growth factor binding protein 4 and retinoid acid receptor α1

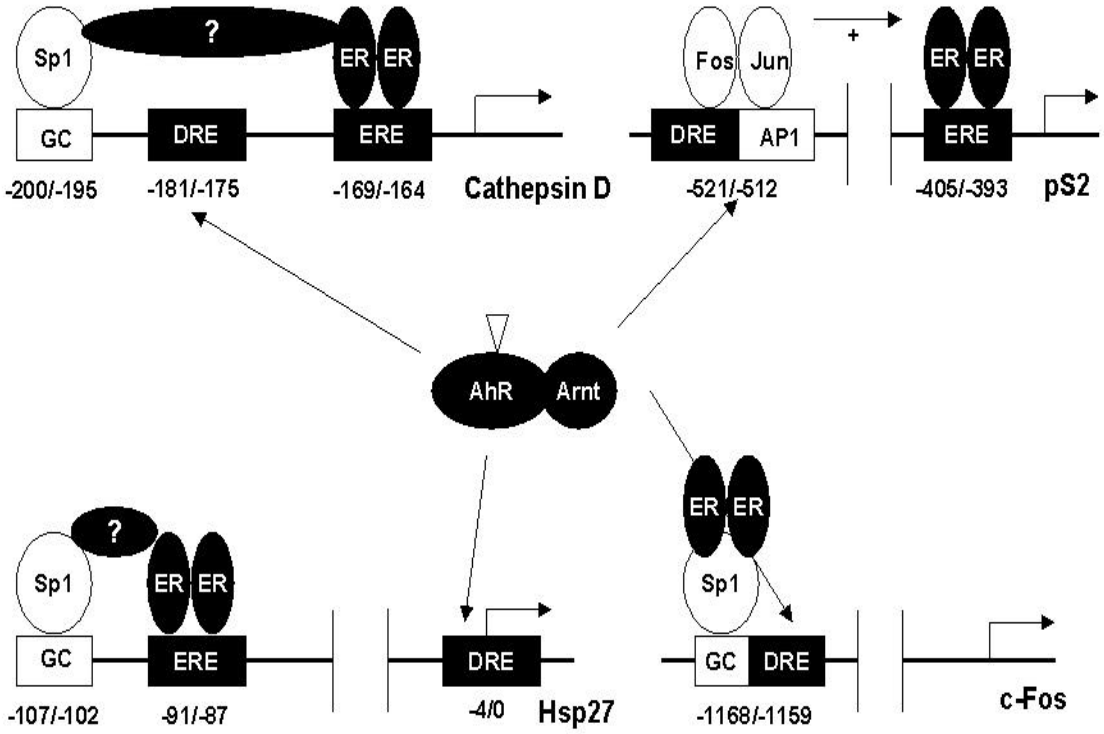


FIG. 4. **Summary of inhibitory AhR-ER crosstalk dependent on direct interactions of the AhR with functional iDREs in the cathepsin D, pS2, Hsp27, and *c-fos* gene promoters.** (94-97)

genes are also induced by E2 in breast cancer cells through ER/Sp1 interaction with GC rich promoters (99-105) ; TCDD also inhibits hormone activation of these genes, however, the mechanisms of these interactions are unknown. TCDD decreases ER levels in the uterus and breast cancer cells, possibly by enhancing the rate of receptor turnover or by inhibiting replenishment of ER. TCDD activates proteosome-dependent degradation of ERα (106). Other drugs that down-regulate ER include phorbol esters, retinoids, cytotoxic drugs such as adriamycin, nonesterified fatty acids such as arachidonic acid, and differentiation-inducing agents such as forskolin.

TCDD induces phase I and phase II enzymes that metabolize E2. Induction of CYP1A1 catalyzes E2 2-, 15α- and 16α-hydroxylation, and CYP1A2 and 1B1 induce hydroxylation of E2 at the C-2 and C-4 positions (107-110). By lowering E2 levels in hormone responsive cells, E2-induced responses may also be decreased.

Studies have demonstrated that regulation of ER action and the activities of other members of the nuclear receptor superfamily (111) are dependent on interactions with other nuclear proteins including both activator and corepressor proteins that are important tissue/cell-specific mediators of nuclear receptor function (112-116). AhR interacts with the coactivators RIP140 (117), ERAP-140 and corepressor SMRT (118). It has been suggested that another mechanism associated with AhR-mediated antiestrogenic responses may be related to competition between the AhR and ER signaling pathways for common coactivators or corepressors. Although there is no direct data illustrating AhR-mediated squelching of E2-induced responses, activation of other steroid hormone receptors does decreases ER-dependent activation of some genes (116).

Diverse AhR agonists

AhR ligands have been separated into two major categories, those that are synthetic in nature (i.e., formed as a result of anthropogenic or nonbiological activity) and those occur naturally (i.e., formed in biological systems as a result of natural processes). The majority of the high affinity AhR ligands that have been identified and characterized to date are members of the first category and include planar, hydrophobic HAHs (such as the polyhalogenated dibenzo-p-dioxins, dibenzofurans, and biphenyls) and PAHs (such as 3-MC, Benzo(a) pyrene, benz[a]anthracenes, and benzoflavones), and related compounds (119-122). The metabolically more stable HAHs represent the most potent class of AhR ligands, with binding affinities in the pM to nM range, whereas the metabolically more liable PAHs bind with lower affinity (nM to μM range). Strucure-activity relationships using a large number of HAHs and PAHs suggests that the AhR ligand binding pocket can bind planar ligands and that high affinity ligand binding appears to be dependent upon key electronic and thermodynamic characteristics of the ligands (122-127). There are many excellent reviews on the physiochemical characteristics and biological/toxicology potency of these “synthetic” HAH/PAH AhR ligands (122,127). An interesting recent development has been the identification of a relatively large number of AhR ligands whose structure and physiochemical characteristics are dramatically different from the “classical” HAH and PAH ligands (128). Classical AhR ligands and CYP1A1 inducers include TCDD, 3,3’,4,4’,5-pentacholorobiphenyl, 2,3,7,8-tetrachlorodibenzofuran, 3-MC, benzo[a]pyrene, β-naphthoflavone. Nonclassical synthetic AhR ligands and/or CYP1A1 inducers include YH439, thiabendazole, omeprazole, SKF71739, (1S. 2R)-(-)cis-1-amino-2-indanol, 5-methyl-2-phenylindole, 2(methylmercapto)aniline, 1-methyl-1-phenylhydrazine, 1,5-

diaminonaphthalene, guanabenz, SRN-P2:109.NH2, 2-(4-amino-3-methylphenyl)benzothiazole (129). High-throughput screening analysis of a combinatorial chemical library using an AhR-responsive reporter gene system (130). has identified numerous novel AhR agonists, including several compounds whose structures contain only a single unsaturated ring (131). Although the majority of the currently identified nonclassical AhR ligands/agonists are relatively weak inducers of CYP1A1 and /or low affinity AhR ligands (when compared with TCDD), the identification of this striking structural diversity of AhR ligands is important because it indicates that the spectrum of synthetic AhR ligands is likely to be much broader than originally anticipated. Thus, attempts to identify endogenous and natural ligands should not be restricted by previous views of the structural requirements for AhR ligands.

The greatest source of exposure of animals and humans to AhR ligands (synthetic and natural) comes from the diet. Numerous studies have described and characterized a variety of naturally occurring dietary chemicals that can directly activate and/or inhibit AhR signaling pathways. The earliest reports of natural AhR inducers came from observations that extracts of vegetables or vegetable-derived materials could induce CYP1A1 activity (132,133). Subsequently, the ability of several dietary plant compounds, such as indole 3-carbinol (I3C) (121,132), 7,8-dihydrorutacarpine (134), dibenzoylmethanes(135), curcumin (136), and carotinoids [e.g., canthaxanthin, astaxanthin, and the apo-carotinoid, β-apo-8'carotenal (137,138)], to competitively bind the AhR and/or induce AhR-dependent gene expression was reported. Conversion of dietary indoles (including I3C) in the mammalian digestive tract to significantly more potent AhR ligands/agonists was also demonstrated. In fact, indolo-(3,2,-b)-carbazole (ICZ), an acidic condensation product formed from I3C (itself a weak AhR ligand), has

perhaps the highest affinity of any “natural” AhR ligand identified to date (~0.2-3.6 nM), and it is a potent inducer of AhR-dependent gene expression in cells in culture(121,132). 3,3’-Diindolylmethane (DIM), another acidic condensation product of 13C, is also an established AhR agonist (139). The formation of relatively potent AhR ligands from precursors that have little or no AhR agonist activity is significant, especially considering that most dietary ligands are themselves relatively weak AhR ligands/agonists. Flavonoids, including flavones, flavanols, flavanones, and isoflavones, represent the largest group of naturally occurring dietary AhR ligands. Although the majority of these natural plant products are AhR antagonists (140-144), numerous agonists, such as quercetin (145), diosmin (146), tangeritin (147), and tamarixetin (140), have also been identified. In addition to interacting with the AhR, many of these flavonoids are also substrates for CYP1A1 (148). These chemicals are widely distributed in dietary vegetables, fruits, and teas (149-151), and flavonoid levels in human blood have been reported to be in the low μM range (152-154), concentrations sufficient to inhibit/activate the AhR. Thus, it is not surprising that crude extracts of a large number of different vegetables, teas, fruits, and natural herbal products have AhR agonist and/or antagonist activity (155,156). Thus, plant-derived materials appear to commonly contain AhR ligands or products that can readily be converted into AhR ligands, and as such, they are perhaps the largest class of natural AhR ligands to which humans and animals are exposed.

The existence of endogenous physiological AhR ligands has been suggested by numerous studies in which the AhR signaling pathway is active in the absence of exogenous ligands. The identification of of nuclear AhR complexes in unexposed cells in culture and tissue slices (157-159), combined with the demonstration that disruption

of AhR expression using antisense resulted in decreased development of mouse blastocysts (160) and alterations in normal cell cycle progression (161,162), supports the existence of endogenous AhR ligands. The ability of hydrodynamic shear stress conditions (163) as well as methylcellulose suspension (92,164) to induce CYP1A1 in cells in culture and of hyperoxia to induce CYP1A1 in rat lungs and liver in vivo (91,165) are consistent with the formation of endogenous AhR ligands in these conditions. The best evidence for a role of the AhR in normal development and physiological/biochemical processes derives from the occurrence of numerous physiological changes and developmental abnormalties in AhR knockout animals (41,166,167). These changes are presumed to result from loss of AhR activation by an endogenous ligand, although the identity of the responsible chemical(s) remains to be determined. Recently, however, a variety of endogenous chemicals have been identified that can bind to the AhR and/or active AhR-dependent gene expression. Although the majority of these chemicals are relatively weak when compared to TCDD, these studies confirm that such ligands do exist. Not surprisingly, these endogenous activators represent several structurally distinct classes of chemicals. These chemicals include indoles, tetrapyroles and arachidonic acid metabolites. Although the role of these chemicals in AhR signaling in vivo remains to be confirmed, their ability to activate the AhR in vitro and in cells in culture suggests that they may also play a role in regulating AhR function in vivo.

AhR antagonists and mechanisms of action

One approach to questions involving the function of the AhR and the role of ligands in regulating this function is to determine how changes in ligand structure alter activity of the protein. A large number of studies have focused on the structure-activity

relationships for agonist activity of potential ligands (8,120,168,169). However, fewer studies have considered the structural basis for potential antagonist proprerties. A number of compounds that bind to the AhR and have weak agonist activity have been shown to antagonize some biochemical and biological responses induced by TCDD. These compounds include several PCB congeners (170), 1-amino-2,7,8-trichlorodibenzo-*p*-dioxin (171), 6-methyl-1,3,8-trichlorodibenzofuran (172), alpha-naphthoflavone (ANF), and other flavone derivatives (173), 1,7- and 4,7-phenanthroline (174), as well as certain benzocoumarins (175) and ellipticines (176-179). Other than conforming to the structural requirements necessary for AhR binding as originally predicted by Poland and Knutson (119), revised by Gillner et al. (121), and recently refined by Waller and McKinney (123), there are no obvious structural similarities among these compounds that would suggest requirements for antagonist activity. This may be related, in part, to different endpoints examined as well as the potential of these compounds to elicit antagonist activity by different mechanisms. Nevertheless, a determination of any consistent structural features necessary for antagonist activity would be valuable in the identification and/or synthesis of potent antagonists that could be used as probes to define the ligand structural features required for AhR agonists. This approach would also delineate structure-dependent relationships among AhR actions, including TCDD-induced altered gene expression and other biological and/or toxicological responses. In an attempt to define such features, a number of ellipticine derivatives and structurally-related compounds were examined. This class of compounds was selected based on the ability of certain of them to bind to the AhR (176), inhibit its transformation to a DNA-binding state (177), and inhibit benzo[a]pyrene hydroxylase activity (176,179), a TCDD-inducible response. Certain ellipticine and

flavone derivatives represent two of the most potent groups of AhR antagonists identified to date.

The structural requirement for substituted ellipticines and flavonoids that exihibit antagonist activity have been investigated using an assay which determines their inhibition of TCDD-induced binding of the AhR to a DRE in a gel mobility shift assay. The results showed that the compounds should fit the hypothetical van der Waals receptor cavity (123) and not contain bulky substituent groups. Secondly, the compounds should be planar and polycyclic aromatic/heteroaromatic. As previously suggested (123), these two properties appear to be required for the best binding activity of agonists or antagonists. Finally, an antagonist should possess an electron-rich heteroatom e. g. ring nitrogen, near or along an otherwise relatively unsubstituted X-axis terminus of the van der Waals cavity (180).

Potent AhR antagonists are planar, with a lateral electron rich center. To further define structural requirements and mechanisms for antagonism, flavone derivatives were synthesized and the most potent flavones contained a 3'-methoxy group and a 4'-substituent having one or more terminal atoms of high electron density (-N_3, -NO_2, or –NCS). Furthermore, these compounds exhibited low agonist activity as indicated by their inability to induce AhR-DRE binding or luciferase activity. Compounds containing bulkier 3' or 4'-substituents, or a 3'-OH group were less potent antagonists, and some were partial agonists. In rat liver cytosol, 3'-methoxy-4'azido- and 3'-methoxy-4'-nitroflavones bound competitively to the AhR, indicating that they bind to the TCDD-binding site. When hepatoma cells were exposed to these flavones, AhR complexes were primarily immunoprecipitable from the cytosol and contained the 90 kDa Hsp. In contrast, AhR in TCDD-treated cells was primarily immunoprecipitable from nuclear

extracts and was associated with Arnt but not the 90 kDa Hsp. Immunocytofluorescence analysis in intact cells further indicated that the potent AhR antagonists inhibited nuclear uptake of AhR and blocked TCDD-dependent down-regulation of AhR. These data indicate that the most potent antagonists bind the AhR with high affinity but cannot initiate receptor transformation and nuclear localization (181).

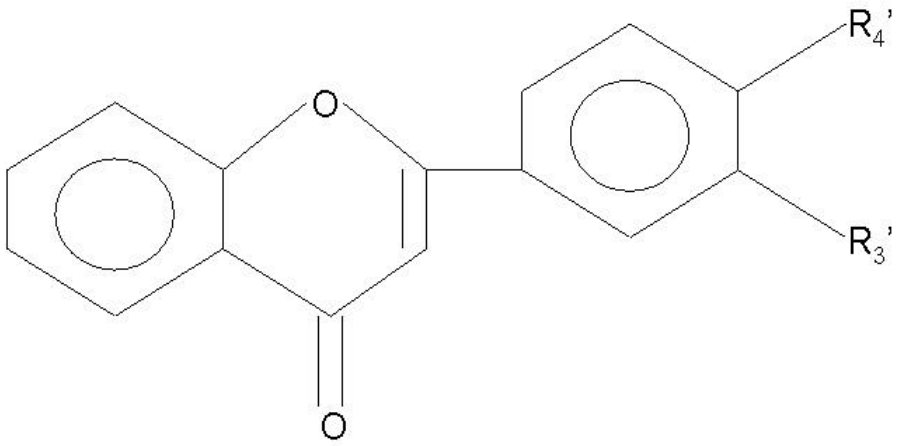
Bioflavonoids and related synthetic analogs exhibit a broad spectrum of biological activity. Naturally occurring plant flavonoids such as naringenin (flavanone), apigenin (flavone), and genistein (isoflavone) which are substituted with hydroxyl groups at the 4' and 3,5,or 7 positions are weak estrogens which bind the estrogen receptor and elicit estrogen-induced responses (182-184). Other studies have reported that naturally occurring polyhydroxylated flavonoids, chalcones, and structurally related synthetic analogs exhibit antimutagenic and anticarcinogenic activities (185-192) and inhibit activities of a number of enzymes including protein kinases (193-196), porcin-5-lipoxygenase (197), ornithine decarboxylase (192), glutathione, reductase (198), P450 isozymes (199), and HIV proteinase (200). Bioflavonoids can also enhance some P450-dependent activities, inhibit or enhance carcinogen-induced tumors or DNA binding (185,191,201-205) and inhibit human platelet aggregation (199). The flavonoid-mediated effects are dependent on numerous factors including the structure of the compound, the target organ or cell, and the response.

The synthetic flavone, 5,6-benzoflavone (β-naphthoflavone), inhibited carconigen-induced tumor formation in the mouse skin model and this activity was related, in part, to inhibition of P450-dependent metabolic activation of carcinogens (185,190-192). Interestingly, β-naphthoflavone binds to the AhR and is often used as a prototypical inducer of AhR-mediated CYP1A1 and CYP1A2 gene expression

(199,206,207). 7,8-Benzoflavone (α-naphthoflavone, αNF) binds with moderate affinity to the AhR and has been extesively characterized as a partial AhR antagonist (208-212). It was shown that in various cancer cell lines, at concentrations $\leq 10^{-6}$ M, αNF inhibited TCDD-induced CYP1A1 gene expression and this was paralleled by decreased formation of the nuclear AhR complex (208). In contrast, αNF was an AhR agonist at a concentrations of 10^{-5} M (213).

Our laboratory has been investigating the modulatory effects of various, 3',4'-substituted flavones on AhR-mediated signal transduction pathways and among the 3'-methoxy-4'-nitro, 4'-amino-3'-methoxy-, 4'-methoxy-3'-nitro-, and 3'-amino-4'-methoxyflavones, 3'-methoxy-4'-nitro- and 4'-amino-3'-methoxyflavone have been extensively characterized as AhR antagonists. These compounds act by inhibiting formation of the nuclear AhR complex (173,180,181,214) (Fig. 5) and similar results have been observed for 2'-amino-3'-methoxy-flavone (215,216). However, many of these substituted flavones are also protein tyrosine kinase inhibitors and cytotoxic at doses > 10 μM (193,215,216). Henry and coworkers (181) investigated a series of 3'-methoxy-4'-substituted flavones and showed that the most active AhR antagonists contained 4'substituents with high electron density (nitro, azido, and thiocyanate). It was hypothesized that this structural feature facilitated critical hydrogen bonding with the AhR. In contrast, 3'-methoxy-4'-aminoflavone was less active as an AhR antagonist (181) than previously observed in our laboratory in breast cancer cell lines (173), and this may be due, in part, to cell context. We also observed that although 4'-methoxyflavone did not inhibit induction of CYP1A1 by TCDD, this compound blocked TCDD-induced transformation of the rat cytosolic AhR (214), and we therefore hypothesized that 3,'4'-dimethoxyflavone (DMF) (Fig. 5), which contains two vicinal

methoxy groups, may be an effective AhR antagonist in breast cancer cell lines despite the lack of a 4'-substituent with high electron density.



	R_4'	R_3'
1	NO_2	OCH_3
2	NH_2	OCH_3
3	OCH_3	OCH_3

FIG. 5. **Structures of 3'-methoxy-4'-nitroflavone (1), 4'-amino-3'-methoxyflavone (2), and 3',4'-dimethoxyflavone (3).**

One of the research objectives of this study was to characterize 3',4'-DMF as an AhR antagonist or agonist.

Selective AhR modulators (SAhRMs)

Prolonged exposure to mitogenic stimulation by estrogen has been considered an important etiologic factor for development of breast and endometrial cancer (217). Tamoxifen has been used extensively for treating ER-positive tumors, however, tamoxifen acts as an ER-agonist in the uterus and increases the risk for endometrial cancer (218). Hence there is a need for development of new classes of antiestrogens that can be used alone or in combination with tamoxifen. Research in our laboratory has demonstrated that TCDD inhibits E2-induced responses in the rodent uterus and mammary tumors (growth inhibition) and in breast and endometrial cancer cell lines through complex inhibitory AhR-ER crosstalk. Inhibitory AhR-ER crosstalk is the basis for development of non-toxic AhR agonists or SAhRMs for modulating action of hormones in a tissue-specific manner. 6-alkyl-1,3,8-trichlorodibenzofuran and substituted DIM represent two structural classes of SAhRMs. These compounds are relatively non-toxic and inhibit ER-positive and ER-negative mammary tumor growth, and synergize with tamoxifen to inhibit breast cancer growth and block tamoxifen-induced estrogenic activity in the uterus (219). Studies also indicate that SAhRMs inhibit prostate cancer cell growth, and there is evidence for inhibitory AhR-androgen receptor crosstalk. SAhRMs represent a novel class of drugs for treatment of hormon-depentent cancers, and combined therapies of SAhRMs plus tamoxifen and other selective ER modulators (SERMs) provides a new approach for treating women with breast cancer (220).

Several studies have shown that alternate-substituted (1,3,6,8- or 2,4,6,8-) 6-alkyl-substituted PCDFs are relatively non-toxic AhR agonists that exhibit potent antiestrogenic activity. Dickerson and coworkers (221) compared the structure-dependent antiestrogenic activity , in the female rat uterus, of a series of 6-alkyl-1,3,8-substituted alkyl PCDFs substituted on two of the four lateral positions (2,3,7,8) to alkyl PCDD/PCDFs that contained three or four lateral substituents. The antiestrogenic potencies of most compounds were similar but the alkyl PCDFs substituted on three or four lateral substituents were more potent inducers of CYP1A1 than the 1,3,6,8-substituted PCDFs and were potentially more toxic than the corresponding alternate-substituted PCDF. The protoype for the alkyl PCDF, 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) (Fig. 6), exhibits low toxicity(52,222,223) but elicits the same broad spectrum of antiestrogenic responses reported for TCDD (52,221). Initial studies in the female rat uterus showed that MCDF was approximately 300-700 times less potent as an antiestrogen and 10,000-100,000 times less toxic than TCDD for the traditional AhR mediated toxic responses (224). MCDF bound competitively with moderate affinity to the rodent cytosolic AhR (EC_{50}=10-100 nM) but was a weak agonist for several AhR-mediated biochemical and toxic responses including induction of lethality, porphyria, teratogenicity, immunotixicity and CYP1A1 and CYP1A2 (225). Moreover, MCDF exhibited partial antagonist activities for several TCDD-induced responses (110). 6-MCDF and 8-methyl-1,3,6-triCDF (8-MCDF) significantly inhibited (>75%) tumor growth in the DMBA-mammary tumor model at a doses of 25, 10 or 5 mg/kg per week and at these doses hepatic CYP1A1-dependent activity was not induced by the alkyl PCDFs. Studies have shown that 6-MCDF can significantly inhibit tumor growth at doses as low as 50 μg/kg/day. MCDF and several alkyl PCDFs also

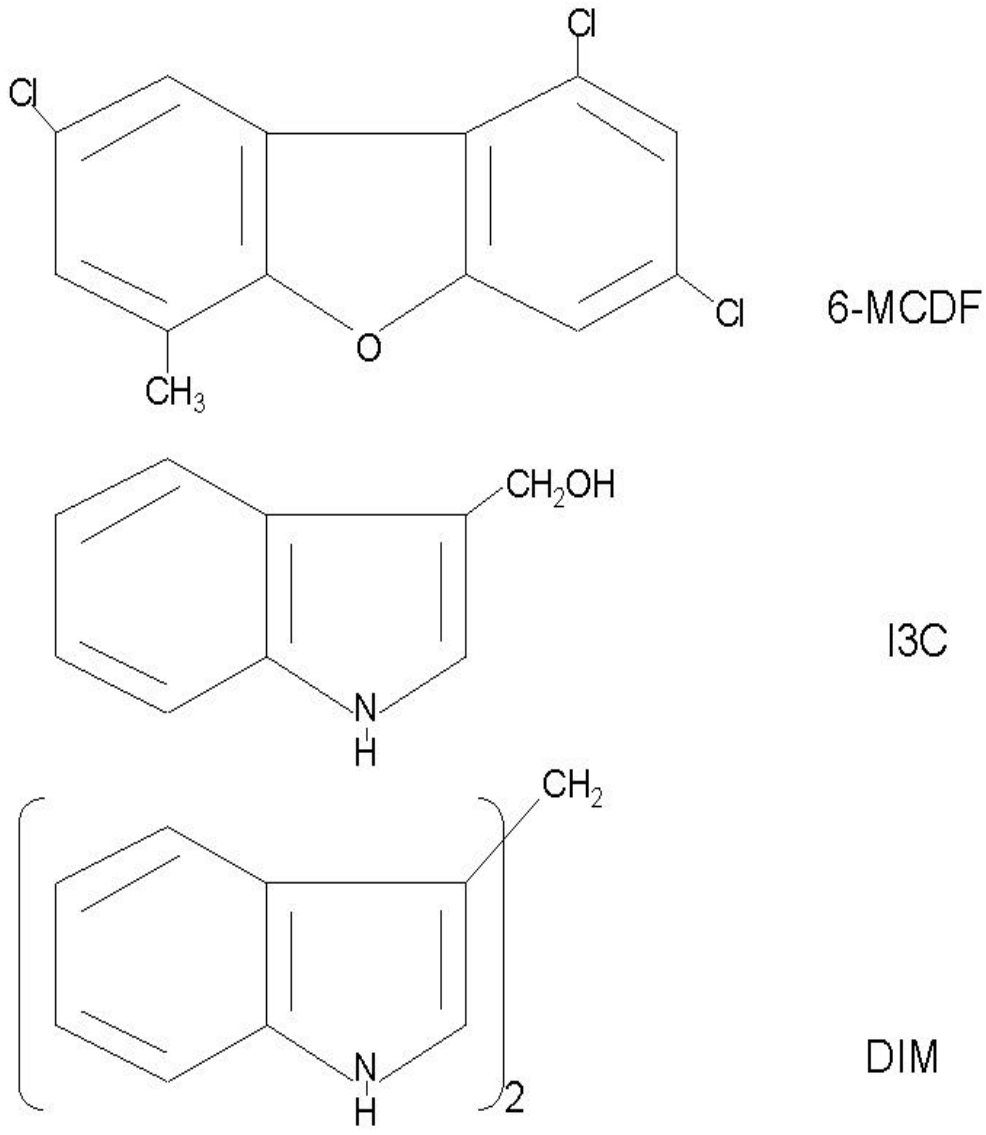


FIG. 6. **Structures of SAhRMs, 6-MCDF, I3C and DIM.**

exhibited antiestrogenic activity in the rodent uterus and inhibited E2-induced uterine cytosolic and nuclear ER and PR levels (226).

Treatment of MCF7 cells with 6-MCDF resulted in downregulation of the ER and inhibition of E2-induced cell proliferation and cathepsin D secretion (227). In a structure-activity relationship study, the antiestrogenic activities of a series of alternate-substituted PCDFs was investigated in the cell proliferation assay and a reporter gene assay using E2-responsive Vit-CAT plasmid. Results showed that MCDF (10^{-5}M) inhibited both E2-induced cell proliferation and CAT activity while the other congeners inhibited at least one response (222). These data indicate that 6-MCDF, which does not exhibit any affinity for the ER, may be a prototype of a novel class of AhR-mediated antiestrogens that could be developed for treatment of breast cancer.

I3C (Fig.6) is an important phytochemical derived from glucobrassicin (3-indolylmethane glucosinolate)-a major component of cruciferous vegetables. I3C exhibits a broad spectrum of antiestrogenic activities in MCF7 cells and anticarcinogenic activities in several organs/tissues in laboratory animals including inhibition of diethylnitrosamine induced neoplastic lesions in SD rats , benzo[a]pyrene-induced forestomach tumors in mice, aflatoxin-B1-induced hepatocarcinogenesis in rainbow trout (228). Several studies have demonstrated that I3C inhibits carcinogen-induced and spontaneous endometrial and mammary tumors in rodents (229-231).

Several studies have suggested that the anticarcinogenic effects of I3C may be partly dependent on its acid-catalyzed condensation products such as DIM (Fig. 6) as well as cyclic (Ctet) and linear (Ltet) tetramers of I3C. In addition, indolo-(3,2,-b)-carbazole, (ICZ) has also been identified as a minor product (232,233).

Both I3C and DIM competitively bind the AhR with low relative binding affinities of 1.0, $7.8x10^{-5}$ and $2.6x10^{-7}$ for TCDD, DIM and I3C respectively (132). In addition, I3C and DIM inhibited TCDD-induced responses including EROD activity, CYP1A1 mRNA levels, formation of nuclear AhR complex and reporter gene activity using an Ah-responsive plasmid construct in T47D human breast cancer cells indicating that both I3C and DIM are partial AhR antagonists (234).

I3C and related indoles modulate P450-dependent metabolism of steroid hormones in a species-, sex- and age-dependent manner. High doses of I3C induced estradiol-2-hydroxylase activity primarily associated with CYP1A2 as well as 6β-hydroxylation of androsterone (52). Studies have shown that ICZ is antiestrogenic in MCF7 cells. ICZ inhibited estrogen-induced cell proliferation, and [^{3}H] thymidine uptake, nuclear PR levels and secretion of procathepsin D (235).

DIM binds the AhR and elicits several AhR-mediated responses including inhibition of human breast cancer cell growth via AhR-mediated pathways (132,139,236). Studies show that DIM (5mg/kg/every day) is a potent inhibitor of DMBA-induced mammary cancer in female SD rats and this was not accompanied by induction of hepatic CYP1A1-EROD activity (237). Induction of CYP1A1 gene expression (a marker of potential toxicity) and antiestrogenic activities by TCDD are usually observed at comparable concentrations in MCF7 cells. However, DIM exhibited antitumorigenic activity at doses which did not induce CYP1A1-dependent EROD activity or alter organ weights or histopathology (237). Furthermore, comparable *in vitro* antiestrogenic responses were observed for TCDD and DIM in MCF7 cells. DIM inhibited E2-induced proliferation of MCF7 cells at concentrations as low as 0.1 μM. DIM also inhibited E2-induced CAT activity in cells transfected with the Vit-CAT plasmid at concentrations of

0.1-10 μM whereas 50-100 μM DIM induced CYP1A1 gene expression. DIM induced the formation of AhR complex in gel mobility shift assays at a concentration (10μM) which did not induce CYP1A1gene expression (237).

The response profiles for 6-MCDF, I3C, and DIM, namely low AhR-mediated toxicity and CYP1A1/CYP1A2 induction and high antiestrogenic activity, suggest that these compounds are a novel class of SAhRMs that selectively inhibits E2-induced responses and may be useful AhR-based drugs for treatment of breast cancer.

Resveratrol interactions with AhR

Resveratrol is a polyphenolic phytoalexin that is found in both free and conjugate forms in high concentrations in grapes, grape juice, and red wine and in other plant extracts (238,239). For example, average concentrations of *trans*-resveratrol (Fig. 7) plus the glucoside conjugate are 3.88 mg/L in red grape juices, whereas lower levels of the corresponding *cis*-isomers (0.85 mg/L) (Fig. 7) have been observed (239). Extracts containing resveratrol have been used in Chinese and Japanese medicine for treating inflammation and cardiovascular disease, and these applications are consistent with many of the biochemical properties observed for resveratrol (240-245). For example, resveratrol inhibits the oxidation of low-density lipoprotein, platelet aggregation, and eicosanoid synthesis implying protection against coronary heart disease, and protects isolated rat hearts from ischemia reperfusion injury. The antioxidant activity of resveratrol is believed to give potential cardio- and neuroprotective effects (246). A role for resveratrol in cardiovascular disease has been proposed (247), but the estrogenic activity of this compound is inconsistent between studies (248-250). Resveratrol also inhibits tumorigenesis in mouse skin and

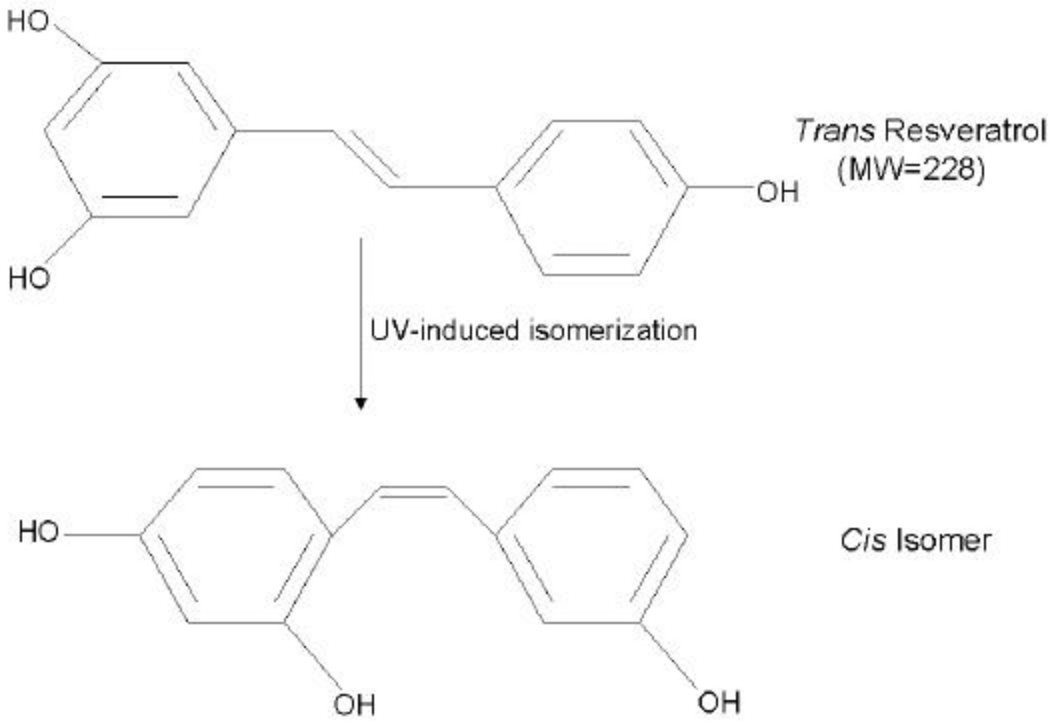


FIG. 7. **Structure of resveratrol (3,4',5 trihydroxystilbene).**

development of preneoplastic lesions in carcinogen-induced mouse mammary glands (251), and these chemopreventive responses may be related to other biochemical effects of resveratrol that include antioxidant responses and inhibition of

cyclooxygenase activity (252-254). Resveratrol has been examined in several model systems for its potential effect against cancer. Its anti-cancer effects include its role as a chemopreventive agent, its ability to inhibit cell proliferation, and its direct effect in cytotoxicity by induction of apoptosis. Moreover resveratrol exhibited therapeutic effects in pre-clinical studies (255). Depending on its concentration, resveratrol can either stimulate (as shown in ER-positive breast cancer and pituitary cells) (256) or inhibit cell proliferation (257-259). At the concentrations used in vitro, resveratrol is predominantly anti-proliferative as demonstrated in a variety of cancer cell lines (260). The mechanism(s) for this growth inhibitory activity of resveratrol could be due to inhibition of ribonucleotide reductase, a complex enzyme that catalyzes the reduction of ribonucleotides into the corresponding deoxyribonucleotides (261). Inhibitors of ribonucleotide reductases, such as gemcitabine (2'-diflluoro-2'-deoxycytidine), are used clinically for their inhibitory effects on DNA synthesis (262). A second possible mechanism for the observed anti-proliferative activity of resveratrol could be related to inhibition of DNA polymerase (263) or ornithine decarboxylase (264) activities. The antioxidant activity of resveratrol could be yet another mechanism for growth inhibition, as a slight pro-oxidant intracellular milieu, an invariable finding in cancer cells (265), is a strong stimulus for proliferation. Resveratrol also inhibits cell proliferation by inducing cell cycle arrest in the G1/S phase (260). Resveratrol has been shown to exert sensitization effects on cancer cells that results in a synergistic cytotoxic activity when used in combination with cytotoxic drugs in drug-resistant tumor cells. Clearly, these studies with resveratrol provide support for the use of resveratrol in human cancer chemoprevention and in combination with chemotherapeutic drugs or cytotoxic factors for treatment of drug-refractory tumor cells (255).

Resveratrol inhibits CYP1A1 expression/levels and CYP1A1-dependent activity in both *in vivo* and *in vitro* models (266-269), and it has been suggested that resveratrol may be an AhR antagonist. Interestingly, other hydroxylated phytochemicals (145,146) such as flavones and the flavonols quercetin and kaempferol, also interact with the AhR, but the former compound is an agonist, whereas kaempferol is an AhR antagonist and blocks induction of CYP1A1 in MCF7 human breast cancer cells (145). Previous studies on resveratrol as an AhR antagonist (146,266,267) showed that although inhibition of CYP1A1 was commonly observed, resveratrol's mechanism of action and its interaction with the AhR were inconsistent. For example, Ciolino and coworkers (146) reported that resveratrol does not competitively bind the AhR but blocksTCDD-induced formation of the nuclear AhR complex; in contrast, Casper and coworkers (267) showed that resveratrol bound the AhR but did not block formation of a nuclear AhR complex in T47D cells cotreated with TCDD. Therefore, our research objective is to reinvestigate resveratrol-AhR interaction as an AhR antagonist.

Our studies confirm that resveratrol inhibits CYP1A1-dependent EROD activity in both MCF7 and T47D breast cancer cells, induces transformation and nuclear uptake of the AhR complex, but does not inhibit induction of reporter gene activity in cells transfected with an Ah-responsive construct containing the –1142 to +2434 region of the CYP1A1 gene promoter. Actinomycin D chase experiments demonstrated that resveratrol decreased CYP1A1 mRNA stability by post-transcriptional processes, thereby decreasing cellular CYP1A1 via AhR-independent pathways.

The AhR antagonist activities of resveratrol is different from that described for the more classical antagonist, 3',4'-DMF. The potential clinical applications for

resveratrol are substantial, however, results reported in thesis should be considered in evaluating specific treatment protocols.

Nuclear receptors and mechanisms of action

Radiolabeled steroid and thyroid hormones of high specific activity were first prepared in the late 1960's, and were used as probes to identify the sites of hormone action (270). It has been known for nearly 30 years that these hormones act via intracellular receptor proteins whose principal target for action is in the nucleus. The receptor proteins were quickly surmised to be regulators of transcription (271-274), and are now known to be part of the nuclear receptor superfamily. This large group of transcription factors includes proteins that mediate the action of the steroid hormones (such as estrogens, androgens, glucocorticoids, mineralocorticoids and the insect steroid hormone ecdysone), as well as the non-steroid hormones (for example, thyroid hormone, vitamin D3 and the retinoids) and receptors that mediate the peroxisomal proliferation response to fatty acids and other factors (111,275-278).

Many other members of the superfamily have been identified by low stringency hybridization analysis; some of the genes thus identified encode proteins that are known to be expressed and have the conserved six-domain structure seen for the hormone receptors (279). It is possible that some of these so-called receptors may act as transcription factors alone, without ligands. To add to the complexity, most classes of receptors within this family contain more than one subtype (i.e., products of closely related genes); sometimes there are also different isoforms (i.e., products from alternate transcription start sites on the same gene) and products of mRNA splice variants. Both the concentration of these receptors and the relative ratio of subtypes and isoforms vary in different target tissues and at different stages of development.

The signature of the nuclear receptor family is a six-domain structure, the most highly conserved portion of which is the small (~70-80 amino acids) domain, C, that is responsible for DNA binding. This domain has been known for some time to have a helix-loop-helix structure containing two zinc atoms, each chelated by four cystein thiols at the start of each helix. Three residues at the start of the first helix in this domain 'read' a five to six base pair code in a DNA hormone-response element; the mechanism of this sequence-specific recognition is becoming increasingly clear through structural analysis of domain C-oligonucleotide complexes by X-ray crystallography (280). The large (~250 amino acid) domain, E, which is moderately conserved across members of the family, is responsible for hormone binding and dimerization, and is critical for the regulation of transcription. The other amino terminal A/B domain, the hinge domain D, and a carboxy-terminal domain F are poorly conserved in length and sequence across the receptor family, and are involved in the modulation of receptor function (Fig. 8).

Recent advances have clarified the various ways in which these nuclear receptors can become activated, as well as some of the molecular details of the modulation of the transcriptional activity of specific genes. The essential and intricate role of the ligand in controlling the regulation of gene transcription by these receptors has also been clarified (281). Although hormones and growth factors that interact with receptors at the cell membrane may ultimately affect gene transcription, they induce multiple-step signal transduction pathways that are extranuclear and direct ligand-dependent activation of nuclear receptors.

The classical picture of gene activation via nuclear receptors requires initial hormone binding to the receptor. The resulting receptor-ligand complex binds as a dimer to a hormone-response element in the promoter region of a regulated gene to

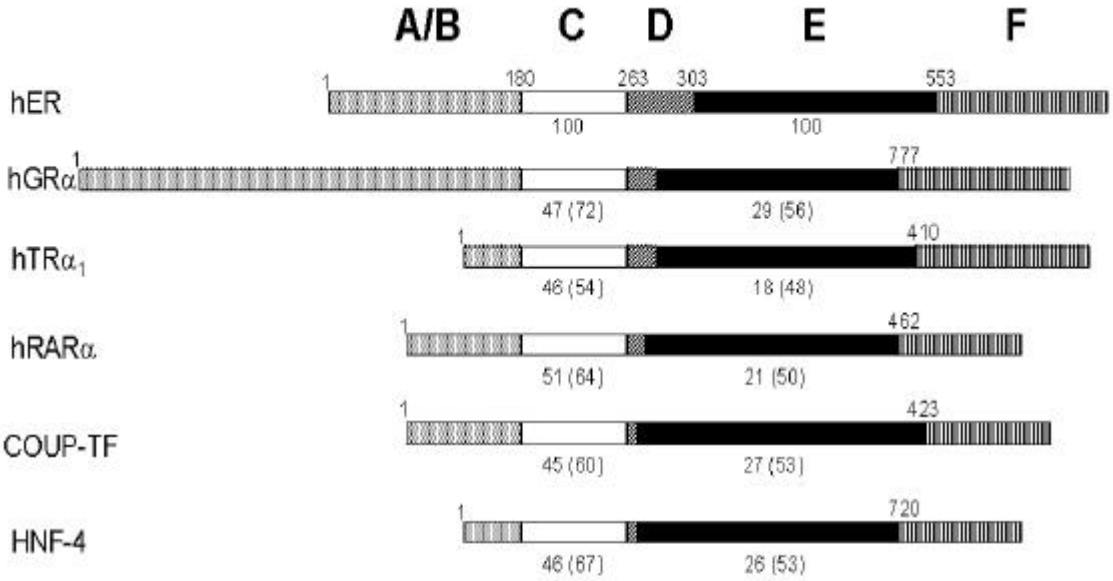


FIG. 8. **Structures of nuclear receptors.** Common domain structure of representative members of the nuclear receptor superfamily, including the human estrogen receptor α (hERα), human glucocorticoid receptor α (hGRα), human thyroid hormone receptor (hTRα$_1$), human retinoic acid receptor α (hRARα), and two orphan receptors COUP-TF and HNF-4. The DNA-binding domain C and ligand-binding domain E are shown with their percent sequence identity (or similarity, in parentheses) compared to hERα.

activate gene transcription. However, this simple model has additional complexities. For example, when estrogen binds to the ER in breast cancer and uterine cells the result is the stimulation of transcription from some early response genes, such as *c-myc*, and genes for growth factors (such as TGFα- or pS2) or growth factor receptors (such as EGF receptor) that are involved in the hormonal stimulation of cell proliferation (282). In contrast, the same ligand-binding event in pituitary and liver cells results in activation of other genes. In the pituitary, the expression of various secreted proteins such as prolactin is increased, whereas in liver levels of vitellogenin, among others, is increased.

Another source of variability is the cellular distribution of the receptor in the absence of ligand. Receptors for certain non-steroidal ligands (e.g.,thyroid hormone and the retinoids) are already bound to their response elements in the absence of ligand (Fig. 9) (283). Ligand binding may strengthen DNA binding, and may alter the conformation of the receptor to enhance transcription. In the absence of ligand, DNA-bound receptors repress gene transcriptional activity (284,285). In contrast, some steroid hormone receptors (e. g., the glucocorticoid receptor) are largely cytoplasmic in the absence of ligand. In the cytoplasm these receptors are complexed with heat-shock proteins, chaperonins, and other proteins such as immunophilins (286). Ligand binding induces release of these proteins and the receptor translocates into the nucleus, dimerizes, and interacts with appropriate hormone response elements (Fig. 9). In such a sheme, the unliganded receptor is not a transcriptional repressor, since it is held in the cytoplasm. And the cytoplasmic localization of unliganded receptors varies with different receptors and cell context.

Another level of variability in receptor action is the content of ligand-activated

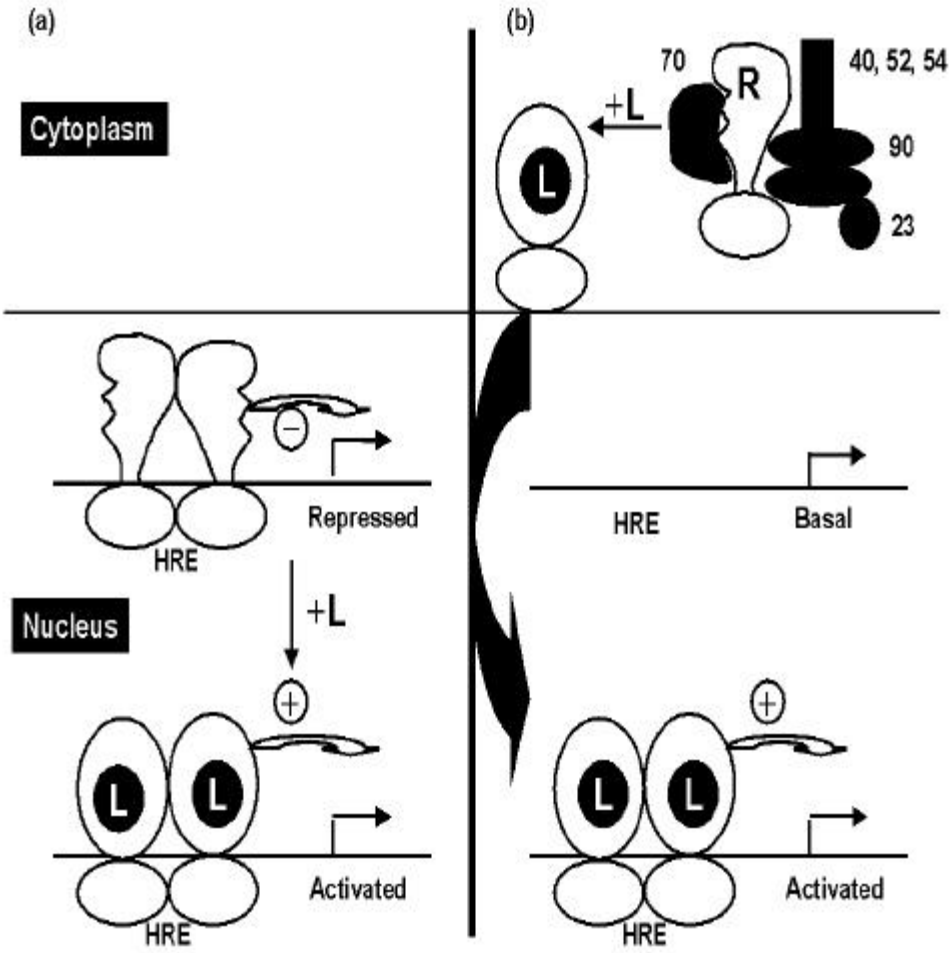


FIG. 9. **The subcellular location of unliganded nuclear receptors affects their modulation of gene transcription.** (a) Unliganded receptors for nonsteroid ligands such as the thyroid hormone and retinoic acid receptors are typically bound as dimers to their hormone response elements (HREs), in the absence of ligand, and act as transcriptional repressors (b) The unliganded receptors for some steroid hormones, such as glucocorticoids, are largely held as monomers in the cytoplasm and are complexed with heat-shock proteins (90, 23), chaperonins (70) and immunophilins (40, 52, 54); in this state, they have no effect on transcription. Ligand binding releases the receptor from the cytoplasmic aggregate, and the activated receptors bind as dimers to the HREs and activate transcription.

receptor dimmers. Non-steroidal hormone receptors such as the hyroid hormone, vitamin D and retinoic acid receptors can either form homodimers or heterodimerize with the retinoid X receptor (RXR) (279,283). The receptor for the insect steroid hormone ecdysone is active only as a heterodimer with the protein ultraspiracle, a homolog of the RXR. The preference of the thyroid, vitamin D and retinoic acid receptors for pairing with themselves or with another partner depends on several factors, including the relative cellular concentrations of the monomeric components (not forgetting the different subtypes and isoforms) and of their cognate ligands. Ligand binding can, in some situations, modulate the formation of specific complexes (287).

Dimerization of steroid hormone receptors primarily involves homodimerization. However, heterodimerization is possible between receptor subtypes (which may have some differences in ligand-binding specificity) and between receptor isoforms (which often have distinctly different transcriptional activities). Examples of subtypes and isoforms that heterodimerize are the glucocorticoid receptor α and β, and progesterone receptor A and B forms, respectively. Receptor dimerization and recpetor stability are important factors for development of pharmaceuticals such as hormone receptor antagonists (e.g., antiestrogens and antiprogestins, for example) (288-291).

Interaction of nuclear receptors with the DNA response elements can also be variable. Response elements are portrayed as consensus sequences of inverted or direct repeats of a defined five- to six-nucleotide sequences, with various spacers between the repeats. However, such response elements found in responsive genes often contain functional nonconsensus sequences; half-sites and multiple repeats of response elements. These elements are often found in complex, upstream-enhancer regions, clustered together or even overlapping with response elements for other known

transcription factors, which may synergize or compete for binding with nuclear receptors. Sequences that flank the core response elements can also affect the DNA binding of these receptors (292). And the structure of the DNA response element, since it affects the recognition between the receptor and the DNA, may also affect the interaction between the receptor and the ligand. Given all these sources of variability, it is not surprising that the response to a specific hormone depends on both the cell in which it is acting and the gene whose activity it modulates (282).

A curious but major difference from the classical scheme for nuclear receptor action is ligand-induced gene transactivation in the absence of direct DNA-binding by the receptor. For these genes the hormone-receptor complex functions by interacting with other DNA-bound transcription factors (293-295) such as fos/jun (AP1) and thus acts as a ligand-dependent co-regulator, rather than a ligand-dependent transcription factor.

Another major deviation from the classical scheme for activation of genes by nuclear receptors is ligand-independent transactivation. In some cells/tissues there is significant crosstalk between different signal transduction pathways that activate transcription. For example growth factors that operate through receptor tyrosine kinases or via cAMP or other second messengers can directly activate nuclear receptors in the absence of ligand (282). Moreover, in some cases, these alternative pathways may synergize with the normal ligand-mediated pathway (296). The molecular mechanisms for these interactions are not well understood, but it is possible that phosphorylation of specific sites on the nuclear receptors may enhance the transcriptional activity of the unliganded receptor (282,297). Several groups (297-299) have shown that ER can be phosphorylated on Ser-118 by mitogen-activated protein kinase, which is activated

through a cascade initiated by binding of EGF to its receptor. Mutation of this serine to an alanine abolishes EGF-mediated activation (298). Substitution of a glutamic acid, which can mimic a phosphorylated amino acid, permits EGF-mediated activation but does not produce a constitutively activated receptor (298). These data suggest that phosphorylation of Ser-118 is necessary, but insufficient, to induce EGF-mediated transcriptional activation and phosphorylation of another sites in ER or alteration of other factors are required.

Once a nuclear receptor is bound to DNA, the subsequent increase in the rate of gene transcription has its own sources of regulatory complexity. First, it is important to recognize that the rate at which a gene is transcribed depends both on the local chromatin architecture, and on the rate at which an active RNA polymerase preinitiation complex can be assembled. Nuclear receptors appear to affect both of these processes, both directly and indirectly via 'transcription intermediary factors' (TIFs)(277,300,301), although their effect on chromatin architecture is poorly understood. There is evidence that DNA-bound nuclear receptors interact directly with some of the proteins comprising the basal transcription machinery, such as TFIIB or TATA-binding protein associated factors (TAFs) (302-304). If they suppress or stimulate a rate-limiting step in the assembly of an active RNA polymerase II preinitiation complex, this would result in repression or activation of transcription. In many cases the relevant interactions between nuclear receptors and basal transcription factors appear to be indirect, and are mediated by various coregulatory proteins.

Estrogen receptor

It was first shown that the effects of the hormone E2 required initial binding to a nuclear receptor ER (305) followed by binding to a unique DNA sequence named ERE

(estrogen response element). The molecular mechanisms of estrogen action required the molecular cloning of the ER cDNA and the development of molecular biological techniques for analysis of transcription. A number of nuclear receptors are known to have subtypes encoded by similar but distinct genes and isoforms that are produced, in part, by alternative splicing. And these include TRα, β; RARα, β, γ; RXR α, β, γ; PPARα, β, γ etc. may be raised. Interestingly, classical steroid hormone receptors (ER, GR, PR, and AR) were long thought not to have subtypes.

However, in 1996, Kuiper et al. (306) found in a rat prostate cDNA library a new type of ER designated as ERβ, and the former (classical) receptor was designated as ERα. Subsequently, the human and mouse ERβs have also been cloned (307,308) and these cDNAs encoded ERβ proteins lacked 53 amino acids at their N-terminus. The first complete human ERβ cDNA was cloned by Ogawa et al. (309) and found to contain 530 amino acids (M_r 59.2 kDa) and Figure 10 shows the structural comparison of the ERα and ERβ. ERβ has an amino acid identity of 96% to ERα in the DNA-binding domain (C), which strongly suggests that ERβ would also recognize and bind ERE, and this has been confirmed by DNA binding experiments. The ligand binding domain (E) has much less homology between ERβ and ERα (53%). However, K_d values for binding of E2 to ERβ and ERα are similar (e.g., 0.6 nM vs 0.2 nM) as determined in different laboratories (306,310). The N-terminal A/B and C-terminal E/F domains are known to have transactivation function 1 (AF1) and transactivation function 2 (AF2), respectively. The fact that these domains are much less conserved suggests that coregulatory proteins interacting with ERβ and ERα are probably different.

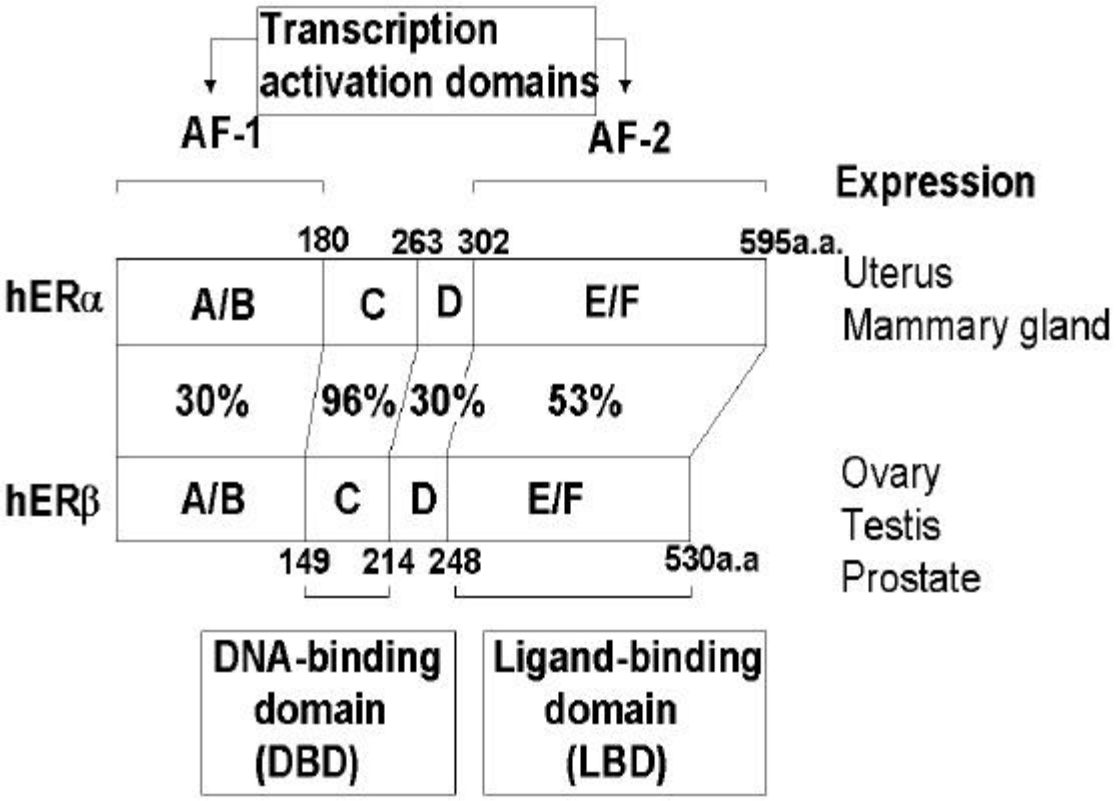


FIG.10. **Structural comparison of ERα and ERβ.** The A to F regions represent classical nomenclature for the structurally similar amino acid sequences of nuclear receptors. The degree (%) similarities of ERα and ERβ in the A/B, C, D and E/F domains are indicated.

Crucial issues for ERα and ERβ are their functional differences and roles in different organs at various stages of development. Tissue distribution of ERα and ERβ is highly variable. ERα is highly expressed in major female organs such as ovary, uterus, vagina, mammary gland and certain areas of CNS especially in hypothalamus. ERβ is not always significantly expressed in those cells except for ovary but higher levels of ERβ are found in male organs and different areas of CNS including some part of hypothalamus and cerebral cortex. More specifically, in the rat, ERβ mRNA was found to be expressed abundantly in prostate and ovaries and less abundantly in uterus, lung, testis, brain and artery (311). In the prostate there are higher levels of ERβ in epithelial cells compared to stromal cells whereas ERα is expressed in both cell types (306). Human ERβ m RNA is more abundantly expressed in testis rather than in prostate (307,310) suggesting that there are also species differences in the distribution of ERβ.

Specific antibodies for the study of ERα and ERβ expression at the protein level have been utilized (312). In ovary, ERβ protein is expressed more strongly in granulosa cells than in theca cells (312). In contrast, ERα is expressed in both cell types and also in interstitial cells (311). There are also ontogenetic changes in expression of ER subtypes; in the pituitary ERβ is abundantly expressed from 12 days of gestation, while ERα appears only after 17 days. In the adult, ERα is widely distributed in anterior lobe of the pituitary gland, whereas ERβ is restricted to certain regions of the anterior lobe (312).

The ultimate role of these receptors has been investigated in the corresponding ER knockout animals. The first ERα knockout (ERKO) mice were produced by Lubahn

et al (313) in 1993. Deletion of ERα was not lethal but both male and female ERKO mice are infertile The basic female reproductive organs such as uterus, ovary and mammary gland were almost normally formed during the pre- and neonatal stages, suggesting compensation by other signaling pathways including ERβ. However, development of these organs in ERKO mice after puberty was severely impaired indicating the requirement of these tissues for ERα and E2 (313). These organs remained immature. Female ERKO mice did not exhibit lordosis when mounted by a male, they became aggressive and frequently showed infanticide (314). It was also observed that male ERKO mice showed abnormalities in spermatogenesis (315) as well as sexual behavior and/or function, indicating that ERα plays a significant role even in male mice (315,316). When the first male human patient expressing an ERα null mutation was found, the major symptoms were the extraordinary long longitudinal bones with epiphyseal unclosure and a lowered bone mass (317). However, this was not observed in male ERKO mice (311). Although they had similarity lowered bone mineral density, they had rather shorter longitudinal bones and this was also prevalent in female mice. This discrepancy may be due to the differences in bone physiology between humans and mice or to the genetic background of the patient. Incidentally, no female patients with either ERα or ERβ deficiency has get been reported. Some aberrant bone metabolism may be expected considering that osteoporosis frequently occurs in postmenopausal women. There is evidence that a transgenic rat expressing a dominant negative ERα can maintain bone density but has a much lower capacity to recover from bone loss when ovariectomized rats are treated with E2. In fact, several alternatively spliced isoforms of ERβ have been reported including $ER\beta_2$ (318) and

$ER\beta_{cx}$ (310), the latter appearing to act as a dominant negative regulator of ERα but not ERβ.

It should be noted that the secondary effects of other factors especially in the hypothalamic-pituitary axis must be considered in analysis of knockout phenotypes. For example, phenotypic changes observed in ERα knockout mice indicate that in adults some of the effects are due to the high circulating levels of lutenizing hormone (LH) which can result in failure of the normal maturational events in the ovary (311).

More recently, ERβ knockout (βERKO) mice and their phenotype has been analyzed (319). Homozygous mice were found to be fertile and did not exhibit abnormal sexual behavior in males or females. Female mice, however, exhibited a reduced ovarian function and fewer pups per pregnancy than normal females. Young male mice showed no apparent abnormalities, however, older mice exhibited hyperplasia of the prostate and bladder. Female ERβ knockout mice tend to have longer and denser bones (320). Estrogen administration inhibits the response to vascular injury in ovariectomized female ERα and ERβ knockout mice as observed in wild –type mice, suggesting that at least one of the ERs or an unidentified ER mediates the vascular protective effects of estrogen (321,322). The reproductive tract in βERKO mice are much less affected than in ERKO mice, however, this does not exclude a role for ERβ in some aspects of reproduction. It is interesting to note that the phenotype of aromatase knockout mice closely resembles that of ERKO (but not βERKO) mice (323). The double-knockout of both ERα and ERβ has also been reported; these animals are infertile and exhibit normal reproductive tract development until pre- and neonatal stages (324). ERα -/- ERβ-/- females exhibit abnormal follicle differentiation and then

follicles resemble seminiferous tubules of the testis. In addition, Sertoli-like cells appear and Mullerian inhibiting substance, sulfated glycoprotein-2 and Sox9 are also expressed. This apparent transdifferentiation indicates that a postnatal sex reversal occurs in the ovaries when both ERα and ERβ are lost (324).

Molecular mechanisms of action of ER

E2 acts by binding to ERα/ER. In addition to the well-known genomic effects exerted by E2 which are mediated by the classical nuclear receptors ERα and ERβ (Fig.11), E2 also induces rapid responses (1-10 min) in different cellular models (325-328). Indeed, both E2 and its cell-impermeant conjugate E2-BSA induce activation of intracellular second messengers such as calcium, nitric oxide formation, activation of kinases such as tyrosine kinases, protein kinase A and protein kinase C, extracellular signal-regulated kinase (ERK) and protein kinase B (PKB) both in neuronal, vascular and bone systems (326) (Fig. 11). Such rapid nongenomic effects are initiated at the plasma membrane level but the nature and characteristics of the mediating receptor is still a matter of debate (329). Studies on both nuclear and membrane receptor in CHO cells transfected with ER(330) and immunohistochemical analysis of pituitary cells (331) support the idea that E2-induced rapid effects are mediated by a small fraction of the nuclear α or β receptors targeted to the cell surface. However, classic ER does not posses either idrophobic domains or potential sites of myristoylation or palmitoylation that will anchor the receptor to the plasma membrane. However it is possible that the nuclear ER is associated with the cell surface by interacting with plasma membrane-associated caveolae and other membrane associated proteins. In fact, a subpopulation of ERα has been localized to plasma membrane caveolae in endothelial cells where it mediates short term effects of E2 through activation of associated endothelial nitric

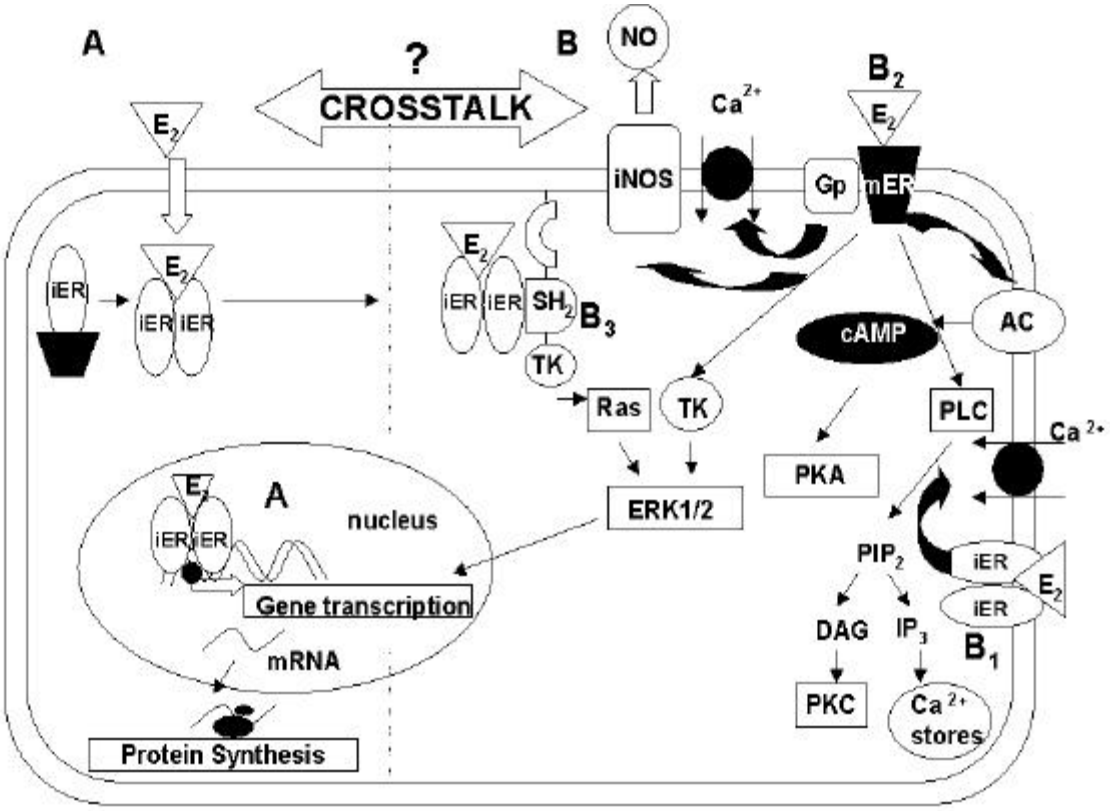


FIG.11. **Hypoyhetical model of estrogen action through different intracellular signaling pathways.** A: genomic pathway through classical cytosolic/nuclear receptors acting as nuclear transcriptional factors; B: nongenomic pathway mediating rapid effects through unusual membrane receptor (B_2) or classical cytosolic/nuclear receptor spanning through plasma membrane (B_1) or acting through multi-proteic complex assosciated to the inner part of plasma membrane (B_3). E2: estrogens; iER: classical cytosolic/nuclear ER; mER: membrane ER; Gp: G protein; iNOS: inducible nitric oxide synthase; NO: nitric oxide; TK: tyrosine kinases; AC: adenylate cyclase; SH_2: Src homology domain; PLC: phospholipase C; PKA: protein kinase A; ERK1/2: ERKs1/2; DAG: diacyl glycerol; IP3: inositol 3-phosphate; PKC:protein kinase C; cAMP: cyclic adenosin monophosphate; PIP2: phosphatidyl inositol biphosphate.

oxide synthase (332).

A different mechanism of nongenomic effects mediated by ER has been hypothesized for E2-induced proliferation in LNCap prostate and MCF7 breast cancer cells. Indeed, estradiol and androgen have been reported to stimulate proliferation in prostate and mammary cancer cells through direct activation of Src at the plasma membrane (333,334). In this model, the androgen receptor (AR) and ERα/β bind to inactive Src forming a ternary complex associated with the inner side of the plasma membrane. This complex subsequently activates Src kinase through Ras/ERK effectors resulting in increased cell proliferation (333,334). This type of nongenomic model for ERα has been confirmed in MCF7 cells hypersensitive to E2 due to prolonged growth in E2-deficient media. In this model ER is sequestered at the plasma membrane and E2 stimulates ERK through formation of ER complexed with Src/Grb2/SOS. Functional experiments in specific cell types that do not express either ERα or ERβ (335-337) also exhibit nongenomic effects of E2 also in the absence of classic ER, suggesting that these rapid effects may be mediated by peculiar isoforms of ER, different from the α and β, that are expressed on plasma membranes (326,338-340) (Fig. 11). Indeed, a novel G-protein coupled membrane ER has been characterized in murine macrophage cell lines (335). Calcium influx stimulated by both E2 and E2-BSA is inhibited by pertussis toxin but not by classic ER blockers such as tamoxifen and raloxifen. Interestingly, the activated receptor is sequestered upon agonist stimulation in an energy and temperature-dependent manner but independently of clathrin and caveolin pathways. A recent study indicates that the rapid effects exerted by estradiol in spermatozoa and testis are mediated by a membrane receptor of about 29 kDa molecular weight that is encoded by a distinct gene consisting of exons 4-8 of the ERα

gene associated with a novel exon called S which is not present in either ERα or ERβ (341). A novel estrogen membrane receptor which mediates estrogen-like effects and has a similar molecular weight has also been identified in uterus, liver and human spermatozoa (339,341-343). Similarity between membrane ER and γ-adrenergic receptor has been also hypothesized (338). A putative ER which negatively regulates T-type calcium-current in mammalian spermatogenic cells has been reported (344), while in Atlantic croaker both estradiol and xenoestrogens reduce production of androgens by Leydig cells in vitro, by binding to estrogen membrane receptors in the testis (327).

A novel functional ER involved in modulation of the rapid effects of progesterone has been identified in human sperm plasma (325,342,343). Indeed, progesterone at physiological concentrations similar to those found in tubal fluid and cumulus oophorous rapidly stimulates signal transduction pathways in human spermatozoa: and these include an increased intracellular calcium levels, acrosome reaction, activation of protein kinase C, ERK and phospholipase A2 (345,346). Western and immunofluorescence techniques have confirmed the presence of specific binding sites for 17βE2 on sperm surface (342,343,345). This 29 KDa membrane receptor shares hormone binding domain homology with nuclear ERα, mediates the rapid and sustained calcium influx stimulated by 17βE2 and interferes with the progesterone-induced calcium response and acrosome reaction (343). Interestingly, the well-known environmental xenostrogens such as bisphenol A (BPA) and octylphenol polyethoxylate do not activate this membrane receptor response in human spermatozoa in vitro (325).

Although ERs in the membrane and nuclear ER act by very different mechanisms (signaling vs. transcriptional transactivation), their biological roles may overlap or be complementary. E2 activates gene transcription using ER from both

receptor pools. One may envision that kinase signaling can rapidly activate transcription, which can then be sustained by subsequent action by nuclear ER which is facilitated by phosphorylation of ER coactivator proteins. Signaling from the membrane may also amplify the actions of the nuclear receptor and play a role in the posttranslational modification of proteins that are induced through transactivation. For example of the anti-apoptotic protein, Bcl-2 is activated by E2, in part through an Sp-1 site contained within the Bcl-2 promoter (100). Moreover, it has recently been shown that the survival function of Bcl-2 can be downregulated by phosphorylation within the "loop domain" of the protein (347). E2 can prevent inactivating phosphorylation of this protein by c-Jun NH_2-terminal kinase (JNK), thereby enhancing breast cancer cell survival (348). Thus the activity and concentrations of this protein are modified by discrete cellular pools of ERs allowing both rapid and prolonged regulation of this cell survival protein. HSP27 and other family members associate with ERs, especially in breast cancer (349). The HSP27 gene is an acknowleged target for nuclear ER transcriptional upregulation (350). Recently, it has been shown that the modulation of HSP27 phosphorylation occurs in response to E2 acting at membrane ER and that this is important hormone-dependent pathway in endothelial cells (351). Thus, membrane and nuclear pools of ERs have different but complementary actions to regulate the short and longer term cell biological consequences of HSP27 function.

Coactivators

Steroid hormone receptors are members of a superfamily of ligand-dependent transcription factors. As such they have a DNA binding domain that recognizes specific target gene sequences along with separate transcriptional activation domains. What sets steroid hormone receptors (and other nuclear hormone receptors) apart from other

families of sequence specific transcriptional activators is the presence of a ligand binding domain (LBD) that acts as a molecular switch in which the hormone induces a conformational change in the receptor to turn on transcriptional activity. Ligand bound steroid hormone receptors recruit coactivator protein complexes that play an essential role in receptor-mediated transcriptional activation. Coactivators function as adaptors in a signaling pathway that transmits transcriptional responses from the DNA bound receptor to the basal transcriptional machinery (352). Coactivators are generally defined as proteins that can interact with DNA-bound nuclear receptors and enhance their transcriptional activation function (353). Hormone receptor agonists induce a conformational change in the AF2 domain that creates a new protein interaction site on the surface of the LBD that is recognized by LXXLL motifs in the p160 family, steroid receptor coactivator (SRC) family of coactivators. In contrast, ER antagonists such as the antiestrogen tamoxifen induce an alternate conformation in AF-2 that occludes the coactivator binding site and recruits corepressors that can actively silence steroid responsive genes. Thus, the cellular availability of coactivators and corepressors is an important determinant in the biological response to both steroid hormone receptor agonists and antagonists (352).

The SRC family of coactivators

Although many nuclear receptor coacitvators have been identified, the SRC family has been the focus of intense study. The first nuclear receptor coacitvator, SRC-1 was cloned by using the PR-LBD as bait in a yeast-two-hybrid screen of a human B-cell cDNA library (354). SRC-1 interacts in a ligand-dependent manner with ER and other NRs and enhances AF-2 dependent transcriptional activation. Recent data also detail the enhancement of ER (355) and AR (356-358) AF-1 activities by SRC-1. In

addition, SRC-1 interacts with the general transcription factors TBP and TFIIB, although the functional consequences of these interactions are unknown (359,360). Furthermore, SRC-1 is able to enhance transcriptional activation mediated by NF-κB, SMAD3, and AP-1 (361-363), supporting a role for nuclear receptor coactivators in multiple intracellular signaling pathways. Subsequent studies have identified two functionally distinct SRC-1 isoforms, SRC-1a and SRC-1e, which contain unique C-termini, suggesting that alternative splicing may also regulate SRC-1 function (364).

The identification of transcription intermediary factor 2 (TIF2) and GR-interacting protein1 (GRIP1) established the SRC family of coactivators (365,366). TIF2 was isolated in a Far-western screen as an ER-and RAR-interacting factor, while GRIP1 was isolated using the GR-LBD as bait in a yeast-two-hybrid screen. TIF2 and GRIP1 share 94% amino acid identity, and represent the human and murine orthologs, respectively. TIF2 and GRIP1 associate in vivo with hormone-bound RAR, ER and PR and coactivate ligand-dependent transactivation. Like SRC-1, GRIP1 also enhances receptor AF-1 activity in addition to that of the AF-2 domain (356). Intriguingly, the inv(8)(p11q13) chromosomal translocation results in a fusion between TIF2 and MOZ gene, which contributes to the pathogenesis of acute myeloid leukemia (AML), suggesting a role for transcriptional regulation by nuclear receptor coactivators in these leukemias (367).

The third member of the SRC family was reported simultaneously by several groups as an RAR-interacting protein (RAC3), a CBP-interacting protein (p/CIP), a hRARβ-stimulatory protein (ACTR), a gene amplified in breast cancer (AIB-1), and a TR-interacting protein (TRAM-1) (368-372). p/CIP represents the mouse homolog, while RAC3/ACTR/AIB-1/TRAM are human isoforms. In addition to coactivating many nuclear

receptors, pCIP enhances the activity of interferon-α and cAMP regulatory element binding protein (CREB), suggesting that this coactivator may be involved in multiple signaling pathways (370). Furthermore, RAC3/TRAM-1 expression can be upregulated by hormone treatment, which represents another possible mechanism by which coactivators potentiate hormone action (373,374).

One of the most important remaining questions to be answered concerning the function of SRC coactivators in vivo focuses on whether or not these three cofactors serve redundant functions. Although all three SRC family members possess similar properties in terms of interactions with nuclear receptors and enhancement of transcriptional activation, several reports suggest that their activities are not completely overlapping and outline differences between SRC-1 and TIF2/GRIP1 versus RAC3/ACTR/pCIP/AIB-1 functions. For example, microinjection of expression plasmids for SRC-1 or NCoA-2, but not pCIP, were able to rescue RAR-dependent activation in SRC-1 immunodepleted cells (370). Also, the relative contribution of each coactivator may depend on cell or tissue type and/or coactivator levels in these cells. RAC3/ACTR/AIB-1 is expressed at high levels in placenta, heart, and HeLa cells relative to TIF2 and SRC-1; thus it may serve a more prominent role in regulating nuclear receptor function in these cells (374). In addition, AIB-1 was cloned as a gene that is amplified in ER-positive BT474, MCF7 and ZR75 breast cancer cell lines (372) and AIB-1 mRNA and protein levels are higher in these cells. SRC-1 and TIF2/GRIP1 are expressed at relatively low levels in these cell lines, suggesting that AIB-1 is specifically involved in the pathogenesis of these tumors. Furthermore, the viability of an SRC-1 knockout mouse may, in part, be due to the observed compensatory overexpression of GRIP1/TIF2 in certain tissues (375). RAC3/pCIP levels are

unchanged in these tissues compared to the wild-type mouse, again supporting a different functional role for this coacitvator versus SRC-1 and TIF2/GRIP1. Finally, a recent study demonstrates that SRC-1 does not colocalize with ERα in rat mammary epithelial cells, but rather is expressed in a distict subset of cells, suggesting that TIF2/GRIP1 or RAC3/ACTR/AIB-1 may be more important for ERα function in these cells (376).

The model of SRC function for regulating nuclear receptor activity is illustrated in Figure 12. Hormone binding triggers nuclear translocation of Type I steroid receptors (ER, PR, AR, GR and MR) and the release of the corepressor complex from Type II non-steroid receptors (RAR, TR and VDR) and subsequent recruitment of an SRC coactivator to the target gene promoter. SRC interacts with the AF-2 domain of each monomer of the dimer via multiple, α-helical nuclear receptor boxes (NR boxes) located in the receptor-interacting domain. SRC is likely complexed with the steroid receptor RNA coactivator (SRA), which enhances AF-1 activity. After initial SRC docking to the receptor, additional coactivators are recruited to the complex. These include CBP/p300, a cofactor of cAMP response element-binding protein (CREB), which uses the NR boxes of the SRC transcriptional activation domain for interaction with coacitvator, and the CBP/p300-associated factor P/CAF. Additionally, direct interactions between CBP/p300 and nuclear receptors and between P/CAF and SRC have also been reported, which may enhance complex formation. Furthermore, SRC recruits coactivator-associated arginine methyltransferase 1 (CARM1) to the target gene via a different domain than that required for CBP/p300 binding. Once this complex is assembled, the histone acetyltransferase (HAT) activities of CBP/p300, P/CAF, and possibly SRC itself, together with the histone methylase activity of CARM1, serve to

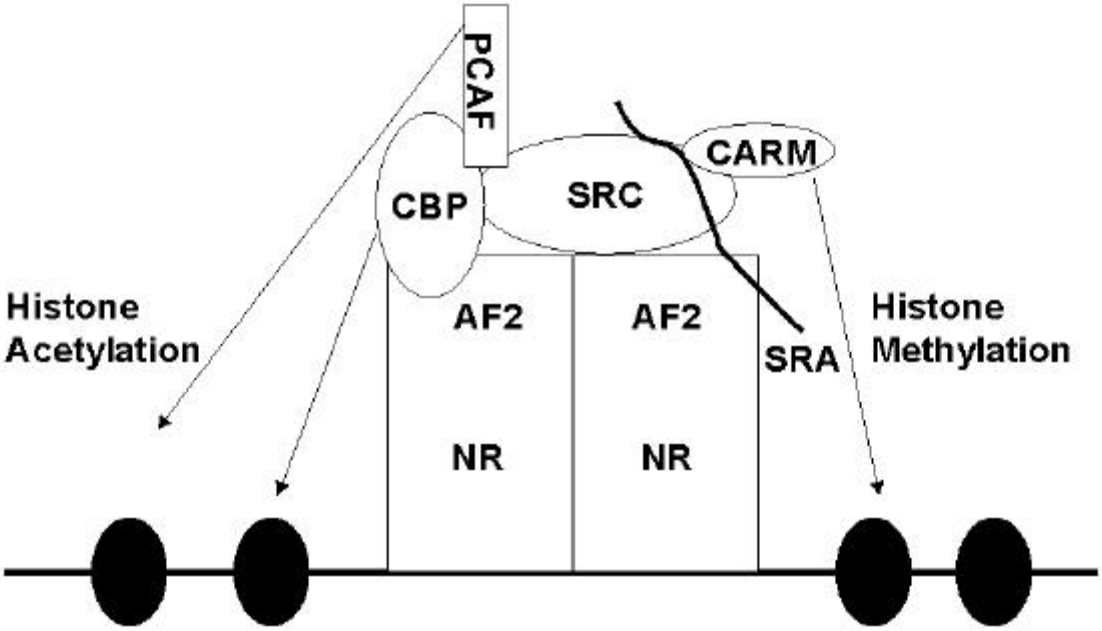


FIG.12. **Model of SRC coactivator function.** Upon binding hormone the nuclear receptor recruits an SRC coactivator through ligand-dependent AF2 interaction, which subsequently results in the recruitment of additional coactivators to the complex. SRC interacts with the receptor and CBP/p300 via LXXLL nuclear receptor boxes. The histone acetylation and methylation activities of various constituents of the coactivator complex facilitate relaxation of the chromatin architecture at the target gene promoter, thereby enhancing transcriptional activation. It should be noted that this is only a general model for a coactivator complex. It is likely that additional cofactors are involved and that different receptors may recruit different components of the complex, thus achieving a level of specificity among receptors and coactivators. NR=nuclear receptor, SRC=steroid receptor coactivator, SRA=steroid receptor RNA coacitvator, CARM=coactivator associated arginine methyltransferase, CBP=CREB-binding protein, PCAF=p300/CBP-associated factor.

remodel the chromatin architecture, thus facilitating the access of additional transcription factors, coactivators such as the vitamin receptor interacting protein (DRIP)/ thyroid receptor associated protein (TRAP) complex, and/or the basal transcription machinery to the target gene promoter to activate transcription (353). Caveats to this model likely exist. The coactivator complex may contain different components depending on the specific nuclear receptor, cell type, or target gene. Different coactivator complexes may determine the specificity among different receptors and is consistent with the potential redundancy among the members of the SRC family. With the intense focus on hormone action and plethora of receptor cofactors that have been cloned, it is likely that additional members of the coactivator complex have yet to be identified. In addition, non-histone substrates for the enzymatically active cofactors may be involved; as described above, CBP/p300 can acetylate non-histone proteins such as ACTR, p53 and TFIIE/TFIIF. Finally, it is possible that receptors recruit single, pre-formed coactivator complexes to the target gene upon hormone binding. Although the precise details of transcriptional activation by nuclear receptors are unclear, the SRC family of coacitvators is critical to receptor function and will continue to warrant investigation on their role in intercellular signaling pathways (353).

The LXXLL motif

The SRC family of coactivators shares a common domain structure, with the most highly conserved region being the N-terminal bHLH-PAS domain (Fig. 13). The bHLH region functions as a DNA-binding or dimerization surface in many transcription factors, including the MyoD family of proteins (20,22). The PAS motif is also found in several transcriptional regulators, including Per, AhR, and Sim. Similar to the bHLH domain, the PAS domain also plays a role in protein- protein interactions and

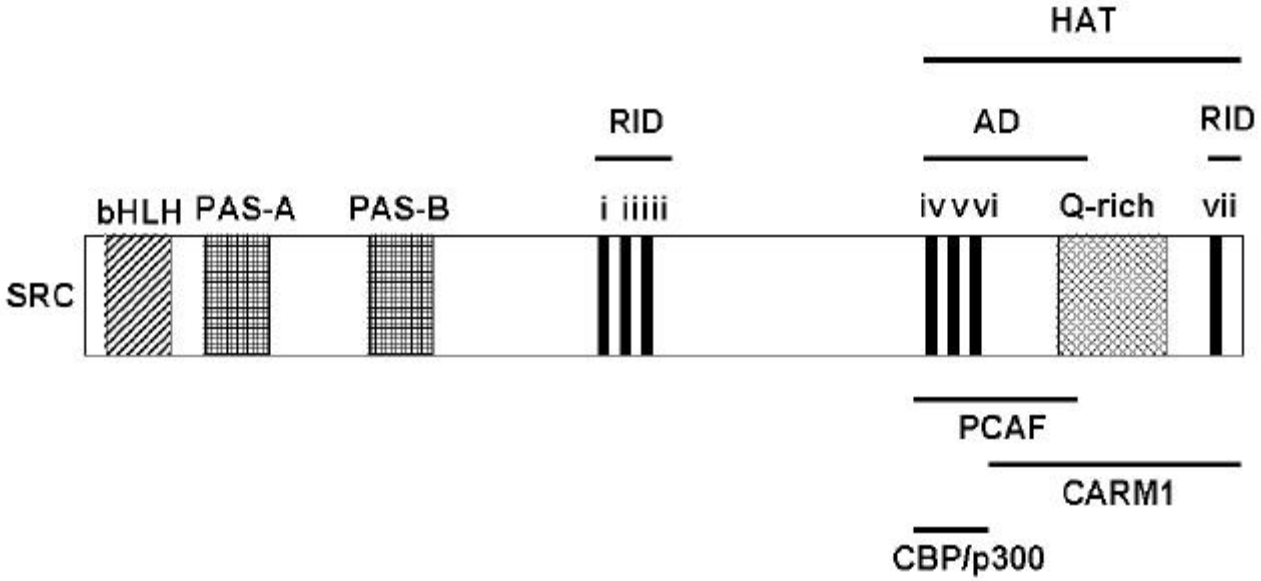


FIG.13. **SRC family domain structure.** Schematic representation of the structural domains of SRC coactivators. The N-terminus contains the highly conserved bHLH and PAS A/B domains. The centrally located receptor-interacting domain (RID) and activation domain (AD) each contains three LXXLL motifs, while SRC-1 contains an additional, non-conserved motif at the C-terminus. The C-terminus contains a glutamine-rich domain. The specific domains for interaction with P/CAF, CBP/p300, and CARM1, as well as the histone acetyltransferase (HAT) domain, are indicated.

dimerization. However, the function of the bHLH-PAS domains of SRC coacitvators remains unknown, although it is likely to mediate intra- or intermolecular interactions. This bHLH-PAS domain is followed by a centrally located receptor interacting domain (RID) and C-terminal transcriptional activation domain (AD). C-terminal AD and RID has HAT activity.

The RID of SRC coactivators mediates ligand-dependent, direct interactions with nuclear receptors (114,374,377). Intriguingly, detailed analysis of the sequence of the RID identified a conserved LXXLL motif (NR box), where L is leucine and X is any amino acid (378). Three such motifs are found in the RID of SRC coactivators, with an additional, non-conserved NR box also present at the C-terminus of the SRC-1 isoform SRC-1a. Site-directed mutagenesis and peptide competition experiments have provided strong evidence for the requirement of these motifs for mediating interactions between coactivators and liganded nuclear receptors (370,378,379). Further support for the role of these motifs in mediating agonist-dependent interactions with nuclear receptors is found in a study in which phage-displayed peptide libraries were screened for peptides that interact specifically with agonist or antagonist bound ER (380). Many peptides isolated with estradiol-bound ERα contained the LXXLL motif, while those isolated with tamoxifen-bound receptor did not. These findings suggest that the activation of ERα by tamoxifen that is observed in some tissues might occur via a different mechanism than estradiol-induced activation, such as through the recruitment of non-LXXLL containing coactivators to tamoxifen-specific surfaces of the ER. Crystallographic and protein structure prediction analyses have indicated that these motifs form amphipathic α-helices with the leucine residues comprising a hydrophobic surface on one face ot the helix. The helix is able to interact with the AF-2 domain of the liganded receptor via a

hydrophobic groove made up of residues from receptor helices 3,4,5 and 12 that is the result of the conformational change induced by hormone binding (370,381-384).

The most interesting aspect of NR box function is the revelation that a receptor-specific code exists, where different nuclear receptors prefer different NR boxes of the RID for interaction with coactivators (379,382,385,386). For example, a 13-aa peptide encompassing GRIP1 motif ii efficiently blocked interaction between GRIP1 and the TRβ-LBD in vitro, while a peptide comprising motif iii was a more potent competitor for GR binding (382). Similarly, yeast-two-hybrid assays demonstrate that mutation of TIF2 motif ii is most deleterious to interactions with PPARα, while a motif i mutation has the greatest effect on the TIF2-RXRβ interaction (386). In all cases, however, mutation of a single motif does not completely abolish coactivator interactions with nuclear receptors, suggesting that multiple NR boxes contribute to the overall, high-affinity binding to the receptor. It is likely that the precise arrangement of multiple motifs and structural nuances of each receptor determines the relative contributions of each NR box to the interaction.

This receptor-specific code has also been analyzed in vivo in terms of transcriptional coactivation of nuclear receptors by SRC-1 via site-directed mutagenesis and antibody microinjection assays (385). The requirement of specific NR boxes for transactivation of reporter genes by different receptors was determined by injecting anti-SRC-1 antibodies into cells along with rescuing plasmids for wild-type or NR box mutants of SRC-1. Anti-SRC-1 IgG completely abolishes transcriptional activation by ER, PR, RAR, TR and PPARγ, while coinjection of wild-type SRC-1 rescues receptor function. Mutation of NR box ii prevented rescue of ER function in SRC-1 immunodepleted cells, while NR boxes ii and iii were required for rescue of RAR and

TR activity and boxes I and ii for PR activity. Furthermore, in the case of PPARγ, different ligands elicited different NR box requirements for SRC-1 coactivation. Troglitazone-bound PPARγ preferred NR box ii over box i, while the opposite was observed in indomethacin-treated cells. Together, these data support receptor-specific LXXLL motif requirements for coactivation function and receptor interactions that account for the presence of multiple NR boxes within SRC coactivators and imply that these motifs do not serve merely redundant functions.

Other determinants that contribute to the specificity of NR box selectivity by different nuclear receptors include residues flanking each NR box. For instance, a chimeric peptide containing the GRIP1NR box iii motif in the context of the flanking sequences of NR box ii competed for TR-LBD binding with a similar potency as the peptide comprising NR box ii (382). Also, using phage-displayed libraries enriched for LXXLL-containing peptides, it was demonstrated that several subclasses of these peptides exist which contain different flanking residues and which vary in their ability to interact with different ER mutants and other receptors (387). Furthermore, it has been shown that the flanking N-terminal amino acids are not essential, while the eight residues C-terminal to the NR box are required for SRC-1 mediated coacitvation of RAR, TR, and ER (385). These studies also revealed additional preferences of ER and RAR for different NR box ii C-terminal amino acids. Intact residues +12 and +13 (where L of LXXLL is +1) are required for SRC-1 rescue of ER activity, while residues at +6, +7, +11 and +13 are necessary for rescue of RAR function. Finally, since most nuclear receptors require two intact NR boxes for coactivator interactions, the spacing between the motifs can also serve as a determinant for recognition. Deletion of 30 of the 50 amino acids between NR boxes ii and iii abolished the ability of SRC-1 to rescue IgG-

mediated inhibition of RAR activity (385). In contrast, proper spacing between NR boxes i and ii was required for coactivation of PPARγ, consistent with the requirement for intact motifs i and ii for maximal PPARγ transactivation.

In addition to SRC-1, a coactivator called proline glutamic acid and leucine-rich protein 1 (PELP1) is also a LXXLL dependent coactivator. For PELP1, a 540 aa region containing seven LXXLL motifs is important for the binding to ERα. Furthermore, transfection of PELP1 (541-1282), which has a deletion of the seven LXXLL motifs, failed to activate the transcription by E2 in ERE reporter assays, suggesting that LXXLL motifs are required for ER binding, and subsequent transactivation coactivated by PELP1 (388).

Coactivators that do not possess LXXLL motifs include CBP/p300-interacting transactivators with glutamic acid [E]/aspartic acid [D]-rich –C-terminal domain 1 (CITED1). The N-terminal region of the conserved CR2 domain which shares a strictly conserved C-terminal transcriptional activating domain among the CITED family and binds to the CBP/p300 transcriptional integrators is required for functional interactions with ERs (389).

LXXLL independent coactivators containing NR boxes have also been reported (390). For example, a coactivator called cell division cycle 25B (Cdc25B) contains a putative NR box (378) in the N-terminal region. The N-terminal fragment (aa 1 to 66) that contains a putative NR box failed to bind ER and Cdc25B with the NR box mutation still enhances ER and GR transactivation similar to that observed for wild-type Cdc25B (390).

DRIP150

Using an affinity column immobilized with the LBD of the vitamin D receptor (VDR), a complex of at least 13 VDR interacting proteins (DRIPs) ranging in size from 30 to 250 kDa were isolated from Namalwa B-cell nuclear extracts (391,392). These proteins selectively bind as a complex to VDR in a 1,25$(OH)_2D_3$-dependent manner. DRIP150 is one of the DRIP complex which exhibits AF2-dependent interactions with the VDR and AR (393) and interactions with the AF1 domain of the GR (394). DRIP150 is also a component of several multiprotein complexes. (i) DRIP150 is part of the DRIP-thyroid hormone receptor associated protein (TRAP) complex, which binds in a ligand-dependent manner to the AF2 regions of the VDR and TR *in vitro*, and is required for transcriptional activation by VDR and TR *in vitro* and *in vivo* (391,395). DRIP150 does not contact AF2 of VDR directly, but rather is brought to the AF2 region of VDR by another member of the complex, DRIP205 which is regarded as a main anchor DRIP (TRAP220/PPAR-binding protein (PBP)) which tethers the complex to the NR (392). (ii) DRIP150 is also a component of the NAT complex involved in transcriptional repression, and has been termed hRGR1 due to its homology to yeast RGR1, a component of the RNA polymerase holoenzyme mediator complex (396). (iii) The DRIP complex is essentially identical to the activator recuited cofactor (ARC) complex, which binds to and is required for transactivation by other transcription factors, such as SREBP-1a, NF-χB p65 and VP16 (397). (iv) DRIP150 is also a part of the SRB/MED-containing cofactor (SMCC) transcriptional regulatory complex (398). DRIP150 is also a member of the smaller cofactor required for SP1 activation (CRSP) complex (CRSP150), which is required for SP1 activation in a purified transactivation system (399,400).

The differences between DRIPs and p160 (SRC) and p300 coactivators are as follows: first, this DRIP complex plays a more global role in transcriptional activation rather than being specific for nuclear receptors. For example, ARC was identified as a coactivator for VP16 and p65 (401), while SMCC enhances p53 activity (398). Secondly, the DRIP complex does not have intrinsic HAT activity.

The mechanism by which the DRIP and SRC complexes contribute to nuclear receptor function is unclear. One possibility involves a two-step model (Fig. 14) in which the SRC complex with HAT activity is first recruited to the nuclear receptor to open up the chromatin network via histone acetylation (402). This would allow access for the large DRIP complex, which would subsequently remodel chromatin, facilitating the organization of the pre-initiation complex or binding of other transcription factors. However, it is also possible that the DRIPs may recruit RNA polymerase to the target gene promoter, for several subunits are homologous to proteins found in Mediator, a transcriptional regulatory complex that associates with RNA pol II (403). In support of this, RNA pol II can be isolated with the SMCC complex at low ionic strength (398). However, this model requires hormone-bound receptor binding to the DRIP complex which then binds the pol II holoenzyme to activate transcription (404). It is also possible that SRC and DRIP functions are not integrated, but exhibit cell-type, promoter, or transcription factor specificity. Specificity may also be the result of the alteration of one or more of the subunits of the complex, depending on the target gene. Overall, it is clear that the DRIP complex is likely involved in the regulation of a broad range of signaling pathways.

The biological roles of DRIPs have been investigated in transgenic mouse models. Using the distrupted and mutated PBP (DRIP205) gene, it was reported that

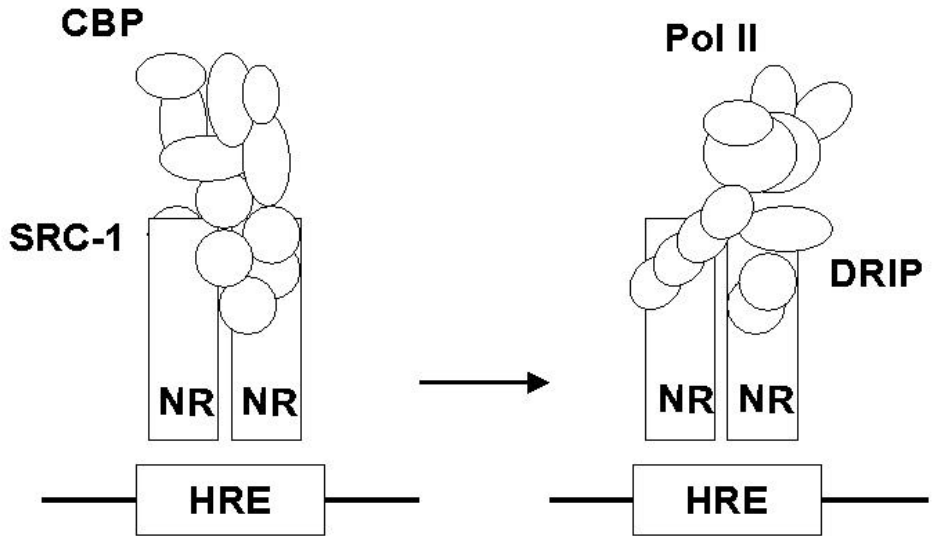


FIG.14. **Model for coactivator assembly.** A two-step hypothesis for recruitment of coactivators involves SRC-1 binding to nuclear receptors and recruits CBP. CBP and associated proteins induce HAT-dependent chromatin remodeling. DRIP complexes then bind receptors and subsequently recruit the RNA polymerase II complex. HRE=hormone responsive element.

DRIPs are essential for embryogenesis and eye-antennal disc development. PBP(+/-) mice are healthy, fertile, and do not differ significantly from PBP(+/+) control littermates. PBP null mutation (PBP(-/-)) in mice is embryolethal at embryonic day 11.5, suggesting that PBP is an essential gene for mouse embryogenesis (405). Drosophila homologues of the transcriptional coactivation complex subunits TRAP240 and TRAP230 are required for identical processes in eye-antennal disc development, suggesting that TRAP240 and TRAP230 act in concert to mediate an unknown developmental signal or a combination of signals (406).

One of the research objectives of this research is focused on the mechanism of DRIP150 coactivation of ERα and ERα/Sp1 in breast cancer cell lines. The studies have investigated the domain of DRIP150 required for functional activity and the results demonstrate the DRIP150 enhances ERα and ERα/sp1-dependent transactivation and coactivation of DRIP150 was LXXLL-independent. Analysis of DRIP150 has identified a unique aa sequence DIPAHLNIFSEVRVYNYRKLILC at aa region 789-811 that is required for coactivation activity.

The model of DRIP150 coactivation for ERα-mediated transactivation is shown in Fig. 15.

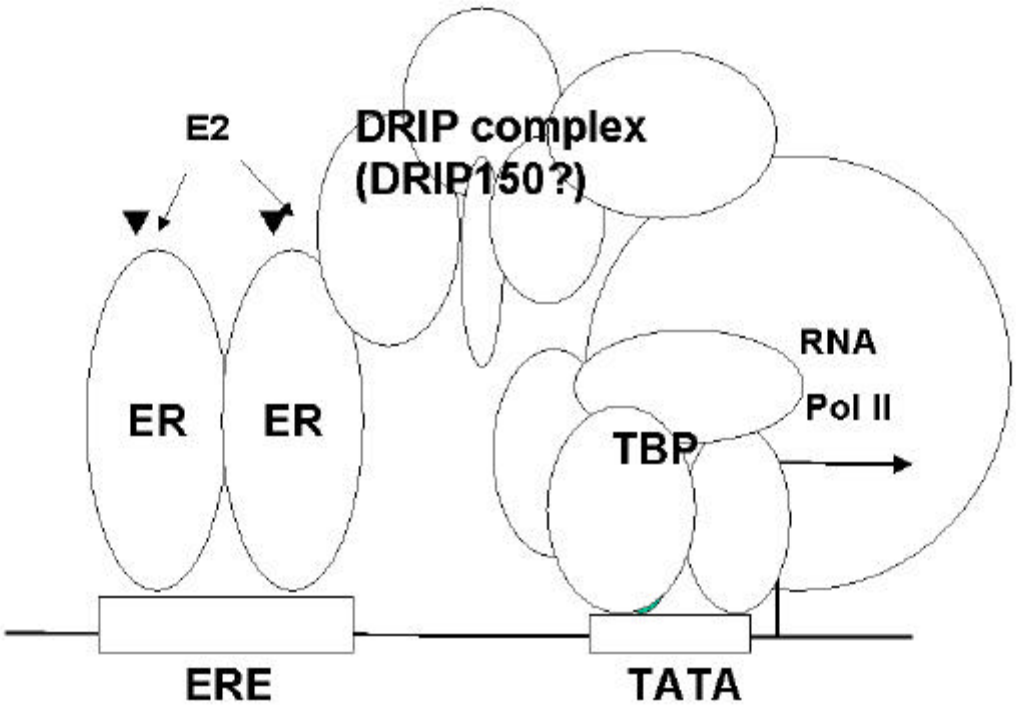


FIG.15. **Model of DRIP150 coactivation for ERα-mediated transactivation.**

CHAPTER II

MATERIALS AND METHODS

3',4-DMF

Cells, chemicals, biochemicals and plasmids

TCDD was prepared by Dr. S. Safe in this laboratory (> 98% pure by chromatographic analysis). 3',4'-DMF was purchased from Lancaster Synthesis Inc. (Windham, NH) (97%) carefully stored in the dark to avoid photodecomposition. MCF-7 and T-47D human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Ethoxyresorufin, 17β-estradiol (E2), Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME F-12) without phenol red, α-minimum essential media (αMEM), phosphate-buffered saline (PBS), acetyl-CoA, and 100X antibiotic/antimycotic solution were purchased from Sigma Chemical Co. (St. Louis, MO). Minimum Essential Medium (MEM) was purchased from Life Technologies (Grand Island, NY). [α-^{32}P]ATP (3000 Ci/mmol) and [^{14}C]chloramphenicol (53 mCi/mmol) were purchased from NEN Research Products (Boston, MA). Poly[d(I-C)] and T4-polynucleotide kinase were purchased from Boehringer Mannheim (Indianapolis, IN). The wild-type double-stranded DRE oligonucleotide 5'-GATCTC CGGTCCTTCTCACGCAACGCCTGGGG-3' and mutant DRE 5'-GATCTCCGGTCC TTCTACATCAACGCCTGGGG-3' were synthesized by Gene Technologies Laboratory (College Station, TX). All other chemicals and biochemicals used in these studies were the highest quality available from commercial sources.

Cell growth

MCF-7 cells were grown as monolayer cultures in MEM supplemented with 10%

fetal bovine serum plus sodium bicarbonate (2.2 g/L), gentamycin (2.5 mg/L), penicillin/streptomycin (10,000 units/L and 10 mg/L), amphotericin B (1.25 mg/L) and 10 μg insulin. T47D cells were grown in αMEM supplemented with 2.2g/l sodium bicarbonate, 5% fetal bovine serum (FBS) and 10 ml antibiotic-antimycotic solution (Sigma). Cells were maintained in 150-cm^2 culture flasks in an air:carbon dioxide (95:5) atmosphere at 37°C. The plasmid pRNH11c contains the regulatory human CYP1A1 region from the Taq I site at -1142 to the BclI site at +2434 fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (kindly provided by Dr. R. Hines, University of Wisconsin at Milwaukee, Milwaukee, WI). The creatine kinase B construct (pCKB) contains the -2900 to +5 promoter insert linked to a CAT reporter gene and was provided by Dr. P. Benfield (Dupont-Merck Pharmaceutical Co., Wilmington, DE). The human ER (hER) expression plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX).

Ethoxyresorufin O-deethylase (EROD) activity

EROD activity was determined essentially as described (407). Trypsinized cells were plated into 48-well tissue culture plates (2×10^5 cells/ml), allowed to attain 60% confluency, and treated with 1 nM TCDD, 0.1 - 10 μM 3',4'-DMF or their combination for 24 hr. After 24 hr, cells were washed by PBS; 185 μl of PBS was added to each well, and cells were incubated in a 37°C water bath for 2 min. The reaction was started by adding 50 μl ethoxyresorufin (1 mg ethoxyresorufin/40 ml methanol) in a 37°C water bath for 13 min. After incubation for 13 min, the reaction was stopped by adding 100 μl of fluorescamine. The 48-well tissue culture plate was scanned for fluorescence measurements using the cytofluor™ 2350 fluorescence measurement system and results for each treatment group were determined as means ± SE for at least three

separate experiments.

Transient transfection assay

Cells were seeded at ~60-70% confluency in 100 mm tissue culture dishes in DME-F12 without phenol red medium supplemented with 5% FBS treated with dextran-coated charcoal (DCC), 1.2 g/l sodium bicarbonate and 10 ml/l antibiotic solution. After 24 hr, cells were transiently cotransfected with 10 μg pCKB and 5 μg hER or 10 μg pRNH11c using the calcium phosphate method. After 6 hr, cells were shocked with 25% DMSO in PBS. Cells were treated with chemicals dissolved in DMSO (0.1%) for 48 hr, and DMSO served as a control. After 48 hr, cells were washed twice with PBS and scraped from the plates. Cell lysates were prepared in 0.1 ml 0.25 M Tric-HCl, pH 7.8 by three freeze-thaw-sonication cycles. Protein concentrations were determined using bovine serum albumin (BSA) as a standard, and aliquots of cell lysate were incubated with 1 μl [^{14}C]chloramphenicol (52 mCi/mol) and 42 μl of 4 mM acetyl-CoA at 37°C. Ethyl acetate was then added; the extract was dried, redissolved in 20 μl ethyl acetate and metabolites separated by thin-layer chromatography (TLC) in 95:5 chloroform:methanol. Following TLC, acetylated products were visualized and quantitated using Packard Instant Imager (Meridian, CT). CAT activity was calculated as the percentage of that observed in cells treated with DMSO alone (arbitrarily set at 100%).

Cell proliferation assay

Cells were seeded at 7.5 X 10^4 cells/well in 6-well plates in media containing 2 ml DME/F12 without phenol red, supplemented with 5% FBS treated with dextran-coated charcoal (FBS-DCC). After 24 hr, the media were changed (5% FBS-DCC) and cells were treated with E2, TCDD, 3',4'-DMF or their combinations for 11 days. The

media was changed and cells were treated with the same chemicals every 48 hr. Cells were then trypsinized, harvested and counted using Coulter Z1 cell counter (Beckman Coulter, Brea, CA).

Preparation of cytosolic and nuclear extracts

Cytosol from male Sprague-Dawley rat liver was essentially prepared as described (Liu et al., 1993). Cells grown in 100 mm Petri dishes and treated with DMSO and the test compounds (3',4'-DMF or TCDD) dissolved in DMSO were harvested and washed twice in 5 ml HE buffer (25 mM HEPES, 1.5 mM EDTA; pH 7.6). Then 0.5 ml of HEGD (25 mM HEPES, 1.5 mM EDTA 10% glycerol, 1.0 mM dithiothreitol; pH 7.6) buffer was added to each plate and cells were scraped and incubated on ice, and then processed with a Dounce homogenizer. Homogenates were centrifuged at 12,000 x *g* for 5 min. Supernatants were discarded, and the pelleted fractions were resuspended in 0.1 ml of HEGD buffer containing 0.5 M potassium chloride (pH 7.6), allowed to stand for 30 min to 1 hr at 4°C, and nuclear extracts were prepared by centrifuging at 12,000 x *g* for 10 min at 4°C. The supernatants representing nuclear extracts were collected and stored in -80°C until used. The protein concentrations were determined using BSA as a standard. Nuclei prepared by this method were found to be inact and were greater than 90% free of extranuclear cellular contamination, as determined by microscopic examination and trypan-blue staining.

Gel electrophoretic mobility shift assay

Nine pmol of synthetic human DRE oligonucleotide was labeled at the 5' end using T4-polynucleotide kinase and [α-^{32}P] ATP. Nuclear extracts from MCF-7 (5 μg) or T47D (2 μg) cells treated with DMSO (control), 5 nM TCDD, 5 μM 3',4'-DMF alone or in combination were incubated in HEGD buffer with poly[d(I-C)] for 15 min at 20°C. The

mixture was incubated for an additional 15 min (20° C) after the addition of [^{32}P]-labeled DNA. Reaction mixtures were loaded into a 5% polyacrylamide gel (acrylamide:bisacrylamide, 30:0.8) and electrophoresed at 110 V in 0.9 M Tris borate and 2 mM EDTA, pH 8.0, and analyzed as described below for the transformed cytosolic AhR-DRE complex. Rat liver cytosol was incubated with different concentrations of 3',4'-DMF alone or in combination with TCDD at 20°C for 2 h. Cytosol (80 μg) in HEGDK buffer [HEDG + 0.4 M potassium chloride) with 1 μg of poly[d(I-C)] was further incubated for 15 min at 20°C. A 100-fold excess of unlabeled wild type and mutant DRE oligonucleotides was added for the competition experiments and incubated at 20°C for 5 min. Following addition of [^{32}P]-labeled DNA, the mixture was incubated for an additional 15 min at 20°C. Protein-DNA complexes were resolved on a 5-6% polyacrylamide gel (acrylamide:bisacrylamide ration, 30:0.8) and run in 1X TBE buffer (0.9 M Tris, 0.09 boric acid, 2 mM EDTA, pH 3.8) at 110 V. Bound complexes were visualized by autoradiography and quantitated by densitometry using the Molecular Dynamics Zero-D software package (Sunnyvale, CA) and Sharp JX-330 scanner (Sharp, Mahwah, NJ), and subjected to autoradiography using a Kodak X-Omat film (Eastman Kodak, Rochester, NY) for appropriate times at -80°C.

Statistical analysis

Statistical differences between different treatment groups were determined using Student's t test or ANOVA (Scheffe's) and the levels of significance were noted ($p < 0.05$). The results were expressed as mean ± SE for at least 3 replicate determinations for each experiment.

Resveratrol

Cells, chemicals and biochemicals

TCDD was prepared in this laboratory (> 98% pure by chromatographic analysis) and resveratrol (99%) was commercially available from Sigma Chemical Co. (St.Louis MO). Trimethoxyresveratrol was quantitatively prepared from resveratrol by methylation of resveratrol using diazomethane in ether/methanol and by monitoring the reaction progress by thin-layer or gas-liquid chromatography. The resulting product was >99% pure by gas chromatographic analysis. 3'-Methoxy-4'-nitroflavone was prepared as described (Lu *et al.*, 1995). Actinomycin D and dehydroisoandrosterone (DHEA), ethoxyresorufin, Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME F-12) without phenol red, α-minimum essential media (αMEM), phosphate-buffered saline (PBS), acetyl-CoA and 100X antibiotic/antimycotic solution were purchased from Sigma Chemical Co. MCF-7 and T47D human breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas VA). Minimum Essential Medium (MEM) was purchased from Life Technologies (Grand Island, NY). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{14}C]chloramphenicol (53 mCi/mmol) were purchased from NEN Research Products (Boston, MA). Poly[d(I-C)] and T4-polynucleotide kinase were purchased from Boehringer Mannheim (Indianapolis, IN). The dioxin response element (DRE), and mutant DRE were synthesized by the Gene Technologies Laboratory at Texas A&M University. The murine CYP1A1 cDNA probe was obtained from ATCC and the plasmid pGMB1.1 was a gift from Dr. Don Cleveland (Johns Hopkins University) and carries the mouse β-tubulin cDNA into the *EcoR I* site of pGMB1.1. Digestion of the plasmid yielded a 1.3-kb fragment that was used to detect β-tubulin mRNA. RNA extraction solution (RNA STAT-60^{TM}) was purchased from Tel-

Test (Friendswood, TX). All other chemicals and biochemicals used in these studies were the highest quality available from commercial sources. The plasmid pRNH11c contains the regulatory human CYP1A1 region from the Taq I site at -1142 to the BclI site at +2434 fused to the bacterial CAT reporter gene (kindly provided by Dr. R. Hines, University of Wisconsin at Milwaukee WI).

Cell growth

MCF-7 cells were grown as monolayer cultures in MEM supplemented with 10% fetal bovine serum (FBS, Intergen, Purchase, NY) plus $NaHCO_3$ (2.2 g/L), gentamycin (2.5 mg/L), penicillin/streptomycin (10,000 units/L and 10 mg/L), amphotericin B (1.25 mg/L) and 10 ug insulin. T47D cells were grown in αMEM supplemented with 2.2g/l sodium bicarbonate, 5% FBS and 10 ml antibiotic-antimycotic solution (Sigma). Cells were maintained in 150-cm^2 culture flasks in an air:carbon dioxide (95:5) atmosphere at 37°C. Media was changed twice per week and when cells became confleunt, cells were trypsinized, passed and reseeded for use in specific studies.

EROD activity

EROD activity was determined as described (407). Trypsinized cells were plated into 48-well tissue culture plates (2 x 10^5 cells/ml), allowed to attain 60% confluency, and treated with 1 nM TCDD, 0.1-10 μM resveratrol for 18-24 hr. For kinetic studies, cells were pretreated with 1 nM TCDD for 18 hr, media was removed and cells were then treated with DMSO, 10 μM resveratrol and 0.5 μM MG132, a proteosome inhibitor for 1, 3, and 12 hr. Cells were washed with PBS and 185 μl of PBS was added to each well and incubated in a 37°C water bath for 2 min. The reaction was started by adding 50 μl ethoxyresorufin (1.25 μg) in a 37°C water bath for 13 min. After incubation for 13 min, the reaction was stopped by adding 100 μl of fluorescamine (60 μg).

Fluorescamine was added to standard wells and the 48-well tissue culture plate was scanned for fluorescence measurement in Cytofluor™ 2350 fluorescence measurement system as described (Willett et al., 1997). Each treatment was carried out in triplicate and results are presented as means ± SE.

Microsomal preparation

Cells grown in 150 mm plates were treated with 1 nM TCDD for 24 hr; cells were then trypsinized, resuspended in 10 ml HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1.0 mM dithiothreitol; pH 7.6) and centrifuged. The supernatant was discarded and 0.5 ml hypotonic HED buffer (25 mM HEPES, 1.5 mM EDTA, 1.0 mM dithiothreitol; pH 7.6) was added to the pellet; the pellet was resuspended and placed on ice for 10-15 min. Samples were centrifuged for 10 min and homogenized using a Teflon pestle/drill apparatus. HEGD buffer (1 ml) was added to the homogenates and centrifuged at 3,000 rpm for 10 min. The resulting supernatant was further centrifuged for 45 min at 4°C, and the microsomal pellet was resuspended in 100 µl Tris-sucrose buffer (38 mM Tris-HCl, 0.2 M sucrose; pH 8.0) and stored at -80°C until used. The protein concentration of the microsomal pellet was quantitated by the method of Bradford (1976). Microsomes were diluted with a cofactor solution containing BSA, NADPH, NADH, and $MgSO_4$ in 0.1 M HEPES to 20 µg protein/5 µl, and 5 µl aliquots were added to 96-well Falcon plates at 0°C, treated with DMSO and different concentrations of inhibitors. Fifty µl of 25 µM ethoxyresorufin was added to each well and incubated at 37°C for 10 min, and the reaction was stopped by addition of 100 µl of MeOH. EROD activity was determined in Cytofluor™ 2350 fluorescence measurement system. The Control experiment was carried out using trimethoxyresveratrol and

diindolylmethane (DIM). Each experiment was determined in triplicate and results are expressed as means ± SE.

Transient transfection assay

The plasmid pRNH11c contains the -1142 to +2434 regulatory region from the human CYP1A1 gene fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. Cells were seeded in 100 mm Petri dishes and grown until 70% confluent. Ten μg of the test plasmid, 2 μg of the β-galactosidase plasmid and 2.5 M calcium chloride were used for transfection. After transfection for 6 hr, cells were shocked using 25% glycerol in PBS and treated with the test chemicals (resveratrol, TCDD or their combination) for 30-48 hr. Cells were washed with PBS and scraped from the plates. Cell lysates were prepared in 1X lysis buffer (Promega) by freeze-thaw cycles with liquid nitrogen. Protein concentrations were determined using BSA as a standard. Each cell lysate (20 μg) was incubated with 0.2 mCi *d*-threo-[dichloroacetyl-1-^{14}C]chloramphenicol and 4 mM acetyl-CoA as substrates at 37°C for 2-5 hr. Following thin-layer chromatography, acetylated metabolites were visualized and quantitated using a Packard Instant Imager (Meridian, CT). CAT activity was calculated as the percentage of that observed in cells treated with DMSO alone and normalized relative to β-galactosidase activity.

Preparation of cytosolic and nuclear extracts

Hepatic cytosol from untreated female Sprague-Dawley rats was essentially prepared as described (Lu *et al.*, 1995). Cells grown in 100 mm petri dishes and treated with DMSO and the test compounds (resveratrol or TCDD) for 30 min to 1 hr were harvested and washed twice in 5 ml HE buffer (25 mM HEPES, 1.5 mM EDTA; pH 7.6). 0.5 ml of HEGD (25 mM HEPES, 1.5 mM EDTA 10% glycerol, 1.0 mM

dithiothreitol; pH 7.6); buffer was added to each plate and cells were scraped, incubated on ice and processed using a Dounce homogenizer. Homogenates were centrifuged at 12,000 *g* for 5 min; the supernatant was discarded and the pelleted fraction was resuspended in 0.1 ml of HEGD buffer containing 0.5 M potassium chloride (pH 7.6). After incubation for 30-60 min at 20°C, the nuclear extracts were obtained by centrifugation at 12,000 *g* for 10 min at 4°C. The supernatants representing nuclear extracts were collected and stored in -80°C until used. Nuclei prepared using this procedure was >90% free of extranuclear material as determined by trypan blue staining.

Gel electrophoretic mobility shift assay

Nine pmol of synthetic human DRE oligonucleotide was labeled at the 5' end using T4-polynucleotide kinase and [A-^{32}P]ATP and incubated with 5 μl 10X phosphorylation buffer, 3 μl polynucleotide kinase (10 μ/μl), 33 μl H_2O and 5 μl [^{32}P]-labeled ATP mixture at 37°C for 30 min. The mixture was purified through TE-10 column (Clontech) and [^{32}P]DRE (120,000 cpm) was used for each sample. Nuclear extracts (2-5 μg) from cells treated with DMSO, 5 nM TCDD, 5 μM resveratrol or TCDD plus resveratrol were incubated in HEGD buffer with 1 μg salmon sperm DNA at 20°C for 10 min to bind non-specific DNA-binding proteins. Excess (100-fold) unlabeled wild type DRE or mutant DRE was added to some samples and incubated at 20°C for 5 min. Experiments with rat liver cytosol (80 μg/incubation) used different concentrations of various compounds which were then incubated at 20°C for 2 hr. The mixtures were further treated with 1 μg of poly[d(I-C)] for 15 min at 20°C, and competition experiments used 100-fold excess of unlabeled wild type and mutant DRE and incubated at 20°C for

5 min. [^{32}P]-labeled DNA was then added to cytosolic or nuclear extracts and incubated for an additional 15 min at 20°C. Reaction mixtures were loaded onto 5% polyacrylamide gel and electrophoresed at 120V in 0.9 M Tris-borate and 2 mM EDTA, pH 8.0. for 2.5 hr. Gels were dried and protein-DNA complexes were visualized by autoradiography using a Packard Instant Imager. The gel was also exposed to a phosphoscreen for 12 hr and visualized by autoradiography using a Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

RNA preparation and northern blot analysis

Cells were plated into 100 mm petri dishes with media containing 5% charcoal-stripped FBS and, when cells reached 60% confluency, they were treated with 1 nM TCDD, 5 μM resveratrol, or their combinations in serum free media for 6 hr. For the kinetic study, cells were pretreated with 1 nM TCDD in serum free media for 12 hr, media was changed and DMSO, DHEA (1 μM) and resveratrol (5 μM) with 5 μg/ml actinomycin D was added to each plate for 2, 6, and 10 hr. After treatment, RNA was extracted from the plates using RNA STAT-60^{TM} purchased from Tel-Test (Friendswood, TX). RNA extracts from different treatment groups were dissolved in nuclease free water, heated at 55-60°C for 15-30 min, vortexed, and quantitated at 260/280 nm. RNA (30 μg) was mixed with 2x sample buffer (20% formaldehyde, 1.65% 1M Na_2HPO_4, pH 6.8, 63.5% formamide and 15% 6X loading buffer), electrophoresed on a denaturing 1.2% agarose gel at 60 V for 2.5 hr, and transferred to a hybond nylon membrane in 1X SPC buffer (20 mM Na_2HPO_4, 2 mM CDTA, pH 6.8) for 36-48 hr. The membrane was then exposed to UV light for 5 min to cross-link RNA to the membrane and baked at 80°C for 2 hr. The membrane was then prehybridized in a solution containing 5X SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 10% dextran

sulfate, 0.1% polyvinyl pyrolidine, 0.1 % ficoll, 0.1% bovine serum albumin and 1% SDS for 18-24 hr at 62°C. The CYP1A1 or β-tubulin cDNA probes were [^{32}P]-labeled using a Boehringer-Mannheim random primer kit. [^{32}P]-labeled cDNA (5 x 10^6 cpm) probes were heated at 100°C for 5 min and cooled on ice for 5 min prior to use and the membrane was hybridized for approximately 24 hr in the prehybridization solution. After hybridization, the membrane was washed at 20°C for 15 min (2X) in a solution containing 1X SSPE and 2% SDS, sealed in a plastic bag. Bands were scanned using a Packard Instant Imager, and the membrane was exposed to phosphoscreen for 1-2 days. CYP1A1 signals were visualized by autoradiography and quantitated using a Storm PhosphoImager. The membrane was then stripped by the washing solution at 62°C for 10 hr, prehybridized for at least 2 hr and rehybridized with 5 x 10^6 cpm [^{32}P]-labeled β-tubulin cDNA probe (in 10 μl) for 24 hr. Washing, visualization and quantitation methods were carried out as described above, and CYP1A1 mRNA was standardized relative to β-tubulin mRNA.

Statistical analysis

Statistical differences between different treatment groups were determined using Student's t test or ANOVA (Scheffe's) and levels of significance were noted ($p < 0.05$). Results were expressed as means ± SE for at least three replicate determinations for each treatment group.

DRIP150

Cell lines, chemicals and biochemicals

The ZR-75 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cells were cultured in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10 % fetal bovine serum (FBS) (Summit

Biotechnology, Fort Collins, CO). Medium was further supplemented with sodium bicarbonate, glucose, Hepes, sodium pyruvate and antibiotic/antimycotic solution (Sigma). Cells were maintained at 37°C with a humidified CO_2:air (5:95) mixture. Phenol-free Dulbecco's modified Eagle's medium/F-12 media, phosphate-buffered saline, and E2 were also obtained from Sigma. [γ-^{32}P] ATP (3000Ci/mmol) was purchased form PerkinElmer Sciences (Boston, MA) and poly [d(I-C)] from Roche Molecular Biochemicals (Indianapolis, IN). Restriction enzymes, 5x luciferase lysis buffer, luciferin and TNT7 in vitro translation kit were purchased from Promega (Madison, WI). Reagents for β-galactosidase analysis were obtained from Tropix (Bedford, MA) and anti-Xpress antibody from InVitrogen (Carlsbad, CA). ER antibodies for gel mobility shift and coimmunoprecipitation assays and ProteinG-plus Agarose were purchased from Santacruz Biotechnology (Santacruz, CA). Recombinant human ERα protein was obtained from PanVera (Madison, WI) and all other chemicals and biochemicals were obtained form commercial sources at the highest quality available.

Oligonucleotides and plasmids

The consensus estrogen-response element (ERE) probe used in gel mobility shift assays was synthesized by the Gene Technologies Laboratory (College Station, TX) and the sequence was 5'-GTC CAA AGT CAG GTC ACA GTG ACC TGA TCA AAG TT-3'. ERα expression plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). Expression plasmids for ERα mutants with deletion of amino acid 1-178 (HE19) and TAF1 containing D538N, E542Q, and D545N mutations were kindly provided by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) and Dr. Donald McDonnell (Duke University, Durham, NC), respectively. cDNA encoding DRIP150 was kindly provided by

Dr. Leonard P. Freedman (Merck Research Laboratories, West Point, PA). The expression plasmid for the GRIP-1 NR-box polypeptide GAL4 fusion protein was also provided by Dr. Donald McDonnell (Duke University). The expression plasmid for the AF1 polypeptide was generated in this laboratory by cloning amino acids 1-180 of ERα into NheI/EcoRV site of pcDNA3.0. pcDNA3.1 –His-LacZ was purchased from InVitrogen (Carlsbad, CA). The $pERE_3$ reporter containing three consensus ERE sites linked to a luciferase gene was created by cloning an oligonucleotide with three ERE elements into BamHI-HindIII cut pXP-2 plasmid (408). ERα-GAL4 fusion protein was constructed as follows. First, the GAL4DBD fusion expression vector pM (CLONTECH,PaloAlto, CA) was digested with BamHI and HindIII, and the oligo sequence GATCCGTGTCTGCAGACGTCGACA was inserted into this digested vector. This oligo was added to create more space between restriction enzymes BamHI and SalI in the polylinker of vector pM, providing a more efficient digestion of these two enzymes when cut simultaneously. This new vector, pM (+10) was then used for construction of the pM-ER plasmid. Primers used for preparing GAL4DBD fusion protein with ER were upper primer CTG TGG ATC CGT ATG ACC ATG ACC CTC CAC ACC AAA and lower primer, TCA TGG TCG ACT CAG ACT GTG GCA GGG AAA CC. After PCR amplification, the ERcDNA fragment was digested with BamHI and SalI and cloned into pM(+10) digested with BamHI/SalI to give pM-ER. The 17m5-GAL4-Luc plasmid containing five copies of the yeast GAL4 recognition motif linked to a luciferase reporter gene was provided by Dr.Patrick Balaguer (INSERM 458, Montpellier, France) and Dr.Tim Zacharewski (Michigan state University, East Lansing, MI).

Cloning of DRIP150 mutants

The △1145-1454 m1(mutant1) of DRIP150 was generated by the KpnI/XhoI

digestion of plasmid pcDNA3.0-DRIP150. After cutting with KpnI/XhoI, the fragments were run on 1% agarose gel, and 3.5kb fraction was eluted and ligated with KpnI/XhoI cut pcDNA3.0 vector. Except for m1, all other clones expressing DRIP150 mutants were generated by PCR amplification and primers used for preparing DRIP150 mutants and GAL4DBD fusion proteins are summarized in Table II and Table III. Xpress tagged m2 and m11 DRIP150 mutants were generated by putting the appropriate fragments into Xpress tagged HisA-pcDNA3.1 vector. PM23aa and related point mutants were generated by inserting DRIP150 23 aa (#789-811 region) and the 23 aa region with the mutated DRIP150 aa #792 (Ala->Pro), #801(Arg->Pro) or double mutant into the pM vector. The 23aa regions were also inserted into the vector pET-28b(+) which is His-tagged in order to express large amounts of this peptide for characterization by X-ray crystallization.

After PCR amplification, cDNA fragments of m2 and m3 were digested with KpnI/EcoRI and cloned into pcDNA3.0 digested with KpnI/EcoRI. cDNA fragments of m4, m5, m6, m7, m8, m9, m10, m11, m12 were digested with KpnI/XbaI and cloned into pcDNA3.0 digested with KpnI/XbaI. The cDNA fragment of Xpress-tagged m2 was digested with ECoRI/XbaI and cloned into HisA-pcDNA3.1 digested with ECoRI/XbaI. The cDNA fragment of Xpress-tagged m11 was digested with BamHI/XbaI and cloned into HisA-pcDNA3.1 digested with BamHI/XbaI. The cDNA fragments of pM23aa, pM23A792P, pM23R801P, and pM23A792P/R801P were also digested with BamHI/XbaI and cloned into pM digested with BamhI/Xba. The cDNA fragment of pET-28b(+)-23aa was digested with BamHI/XhoI and cloned into pET-28b(+) digested with BamHI/XhoI.

TABLE II
Primers used for preparing DRIP150 mutants

Constructs	Primers used	Vectors cloned In
△789-1454 m2	Upper primer, GGC TAA CTA GAG AAC CCA CT Lower primer, AAA GAA TTC CTA TGG TAG AGA ACG TGC AAA TTC	pcDNA 3.0
△325-1454 m3	Upper primer, same as m2 Lower primer, AAA GAA TTC CTA CAC AAG GTC TCC CCA CCG TTC	pcDNA 3.0
△977-1454 m4	Upper primer, AGA GCT CTC TGG CTA ACT AGA GAA CCC ACT Lower primer, AAA TCT AGA CTA CCT TCT TCG AGC ATC CTG ATT G	pcDNA 3.0
△886-1454 m5	Upper primer, same as m4 Lower primer, AAA TCT AGA CTA GAG TTT GTT GAT GGC ATT TA	pcDNA 3.0
△870-1454 m6	Upper primer, same as m4 Lower primer, AAA TCT AGA CTA TAA TAA CTG AAC CAC ATT	pcDNA 3.0
△865-1454 m7	Upper primer, same as m4 Lower primer, AAA TCT AGA CTA ATT TGG TGT TTT GTT GAA CAT	pcDNA 3.0
△1-754 & △886-1454 m8	Upper primer, AAA GGT ACC GCC GCC ATG GAG CCT GTT GGT GGT AGA AAG GTG GTT GAA Lower primer, same as m5	pcDNA 3.0
△850-1454 m9	Upper primer, same as m4 Lower primer, AAA TCT AGA CTA ATT GTG ACA GTT ACT GCA ACC	pcDNA 3.0
△827-1454 m10	Upper primer, same as m4 Lower primer, AAA TCT AGA CTA CGA ATT CCA TTG GAT ACT AAT	pcDNA 3.0
△812-1454 m11	Upper primer, same as m4 Lower primer, AAA TCT AGA CTA ACA CAA GAT AAG TTT TCG GTA	pcDNA 3.0
△1-77 & △865-1454 m12	Upper primer, AAA GGT ACC GCC GCC ATG GAT GTG GAA AGG AAA ATA GAA ATA GTG CAG Lower primer, same as m7	pcDNA 3.0
△789-1454 Xpress-tagged m2	Upper primer, AAA GAA TTC TGA CCG CCG CCA TGG CCC CAG TGC AGC TGG AGA ACC ACC Lower primer, AAA TCT AGA CCT ATG GTA GAG AAC GTG CAA ATT C	HisA-pcDNA 3.1
△812-1454 Xpress-tagged m11	Upper primer, AAA GGA TCC AGA CCG CCG CCA TGG CCC CAG TGC AGC TGG AGA ACC ACC Lower primer, AAA TCT AGA CCT AAC ACA AGA TAA GTT TTC GGT A	HisA-pcDNA 3.1

TABLE III
Primers used for preparing GAL4DBD fusion proteins

Constructs	Primers used	Vectors cloned in
pM23 aa	Upper primer, AAA GGA TCC GTA CCG CCG CCA TGG ACA TAC CTG CTC ATC TAA ATA Lower primer, AAA TCT AGA ACT AAC ACA AGA TAA GTT TTC GGT AAT	pM
pM23A792P	Upper primer, AAA GGA TCC GTA CCG CCG CCA TGG ACA TAC CTC CTC ATC TA Lower primer, same as PM-23 aa	pM
pM23R801P	Upper primer, same as PM-23 aa Lower primer, AAA TCT AGA ACT AAC ACA AGA TAA GTT TTC GGT AAT TAT AAA CAG GAA CTT CTG AGA	pM
pM23A792P/R801P	Upper primer, same as A792P m PM-23 aa Lower primer, same as R801P m PM-23 aa	pM
PET-28b(+)-23aa	Upper primer, AAA GGA TCC GAC CGC CGC CAT GGA CAT ACC TGC TCA TCT AAA TA Lower primer, AAA CTC GAG ACA CAA GAT AAG TTT TCG GTA AT	pET-28b(+)

Transient transfection assays

ZR75 breast cancer cells were grown and maintained in RPMI1640 supplemented with 10% FBS. Cells were seeded onto 12-well plates in phenol-free Dulbecco's modified Eagle's medium/F-12 supplemented with 2.5 % charcoal-stripped FBS. After 18 hr, cells were transfected by the calcium phosphate method with 1 μg of $pERE_3$ reporter plasmid, 0.25 μg of a CMV β-gal expression plasmid, the appropiate ERα expression plasmid, and the appropriate DRIP150 expression plasmid. After 6-8 hr, cells were shocked with 25% glycerol in phosphate-buffered saline (PBS) for 75 sec, rinsed once with PBS, and treated with either DMSO or 10 nM E2 in Dulbecco's

modified Eagle medium/F-12 plus 2.5 % charcoal-stripped fetal bovine serum for 36 hr. Cells were harvested by scraping the plates in 100 μl of 1x lysis buffer (Promega). Thirty five μl of the cell lysate was used for performing luciferase assays on a Lumicount Luminometer (Packard Instrument Co.). Thirty five μl of the cell lysate was used for determining β-galactosidase (β-gal) activity on a luminometer. Normalized luciferase values were calculated by dividing the luciferase by the β-gal activities for a given sample. Results are expressed as means $\pm$ S.E. for at least 3 separate experiments for each treatment group and compared with the DMSO control group (arbitrarily set at 1) for each set of experiments.

Gel elctrophoretic mobility shift assays

Five picomoles of synthesized ERE was labled at the 5' end using T4-polynucleotide kinase and [γ-^{32}P] ATP. Plasmids containing the DRIP150, m1, m2 and m3 cDNAs were used to in vitro transcribe and translate the corresponding protein in a rabbit reticulocyte lysate system (Promega). Three μl of recombinant human ERα (500 fmol) was mixed with 3μl of BSA (500 ng/μl), 2 μl of poly(dI-dC) (1 μg/μl), 5 μl of 5x binding buffer (20 mM Hepes-5% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA) and 1 μl of E2 ($3.5x10^{-7}$M) to give final concentration $2.5x\ 10^{-8}$ M E2 and incubated on ice for 15 min. In vitro translated DRIP150, m1, m2 or m3 were then added to the above mixture and incubated on ice for 5 min. To balance the volume, in vitro translated $PcDNA_{3.0}$ was also added. For supershift experiments, 2μl of normal IgG or ER antibody was added to the mixture after 5 min and then incubated on ice for an additional 5-10 min and 5 μl of [^{32}P]-labeled ERE probe (120,000 cpm) was added to the reaction mixture, giving a final volume of 25 μl.The mixture was incubated at 20°C

for 15 min. Samples were then loaded onto 5% polyacrylamide gel and run at 110V in 0.09 M Tris, 0.09 M borate, 2 mM EDTA (pH 8.3) for 2.5 hr. The gel was dried, exposed to a phosphoscreen for 12 hr and protein-DNA binding was visualized by autoradiography using a Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Coimmunoprecipitation assays

Two hundred μl of reticulocyte lysate was mixed with 40 μl of Protein G plus Agarose and 17 μl of ER antibody and shaken for 1 hr at 4°C to preclear ER expressed in the reticulocyte. After 1 hr, the mixture was centrifuged at 1,500xg for 5 min, and the supernatant was used to in vitro translate DRIP150, ER and $PcDNA_{3.0}$. DRIP150, ERα and $PcDNA_{3.0}$ were in vitro translated using [^{35}S]-methionine in a rabbit reticulocyte lysate system (Promega, Madison, WI) and 10 μl of the in vitro translated [^{35}S]-DRIP150 was mixed with 2 μl of in vitro translated [^{35}S]-ER and incubated with $13.2x10^{-7}$M E2 to give a final concentration of 100 nM E2 on ice for 15 min. Ten μl of ERα antibody was then mixed with Protein G plus Agarose (1:2 ratio) and added to the above mixture and incubated for 3 hr at 4°C with shaking every 30 min. For samples not containing [^{35}S]-DRIP150, only [^{35}S]-ER (2 μl) was mixed with ER antibody-Protein g plus Agarose mixture and incubated for 3hr at 4°C as described above. PBS (1 ml) was then added to each sample, and shaken for 30 sec then centrifuged at 1,500xg for 5 min. After centrifugation, the supernatant was discarded carefully and the pelleted fraction (100 μl) was mixed with 20 μl of 1x sample buffer (50 mM Tric-HCl pH 6.8, 2% SDS, 0.1 % bromophenol blue, 10 % glycerol and 100 mM DTT) containing β-mercaptoethanol. The sample was then boiled for 5 min, loaded onto SDS-polyacrylamide gel and run at 150 V for 4 hr. The gel was dried, exposed to a phosphoscreen for 3 days and proteins

were visualized by autoradiography using a Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Western immunoblot assays

COS-7 cells were seeded in 6 well plates at a concentration of 200,000 cells/well in phenol-free Dulbecco's modified Eagle's medium/F-12 with 2.5 % charcoal-stripped FBS. After 24 hr, the media was removed and serum and antibiotic-free phenol-free Dulbecco's modified Eagle's medium/F-12 was added to the wells. X-press tagged DRIP150 m2 and DRIP150 m11 were then transfected using the lipofectamine transfection method (InVitrogen, Carlsbad, CA). After 6 hr, the media was removed and phenol-free Dulbecco's modified Eagle's medium/F-12 with 2.5% charcoal-stripped FBS was added and cells were incubated for 36 hr. Cells were then harvested in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10%(V/V) glycerol, 1% TritonX-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 50 mM phenylmethylsulfonyl fluoride, 50 mM sodium orthovanadate), placed on a rocker at 4°C to extract soluble protein, and centrifuged at 14,000 xg for 10 min at 4°C. Protein was quantitated and an equal amount of proteins (150 μg) diluted with loading buffer was boiled and loaded on 10% SDS-polyacrylamide gel. Samples were electrophoresed at 150-180 V for 3-4 hr. For samples containing in vitro translated Xpress-tagged m2 and m11, Xpress tagged DRIP150 m2 and DRIP150 m11 were in vitro translated in a rabbit reticulocyte lysate system (Promega), diluted with loading buffer, boiled, loaded on 10% SDS-polyacrylamide gel, and electrophoresed at 150-180 V for 3-4 hr. The separated proteins were transferred (in a buffer containing 48 mM Tris-HCl, 29 mM glycine, and 0.025 % SDS) to PVDF membrane (Bio-Rad, Hercules, CA). Specific proteins were detected by incubation with mouse monoclonal anti-Xpress antibody (1:5000 dilution)

for 4 hr, rinsed with distilled water (3x) followed by blotting with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5000 dilution) for 1.5 hr. The membrane was then washed with PBS-Tween20 (0.05%) and blots were exposed to chemiluminescent substrate (ECL) (NEN Life Science Products, Boston, MA) and placed on Kodak X-Omat AR autoradiography film. The detected bands were scanned using a Sharp JX-330 scanner (Sharp Electronics Corp., Mahwah, NJ).

Statistical analysis

Statistical differences between different treatment groups were determined using Student's t test or ANOVA (Fisher's Protected LSD) and the levels of significance were noted ($p < 0.05$). The results were expressed as mean $\pm$ SE for at least 3 replicate determinations for each experiment.

CHAPTER III

RESULTS*

Effect of 3',4'-DMF on EROD and CAT acivity

3',4'-DMF (0.1 - 1.0 μM) alone did not significantly induce EROD activity in MCF-7 or T47D breast cancer cells, whereas the AhR agonist TCDD was a potent inducer in both cell lines (Fig. 16). In cells cotreated with 1 nM TCDD and 0.1 - 10 μM 3'4'-DMF, there was significant inhibition of induced EROD activity by 3',4'-DMF at the 1.0 and 10 μM doses, and almost complete inhibition was observed at the highest concentration of 3',4'-DMF. To ensure that this inhibitory response was not just due to direct interactions of 3',4'-DMF with CYP1A1 protein, the effects of 3',4'-DMF on TCDD-induced CAT activity in MCF-7 or T47D cells transfected with Ah-responsive pRNH11c were also investigated. The results (Fig. 17) show that 3',4'-DMF alone did not exhibit AhR [1] agonist activity, but 3',4'-DMF significantly inhibited TCDD-induced CAT activity in MCF-7 and T47D cells cotreated with 3',4'-DMF + TCDD. Previous studies with AhR agonists that inhibit induction of CYP1A1 by TCDD have shown that some of these compounds such as alkyl substituted PCDFs and I3C/DIM exhibit AhR agonist activity in breast cancer cell lines and inhibit E2-induced growth and transactivation (234,237,409).

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*Part of the data reported in this chapter is reprinted with permission from 3',4'-Dimethoxyflavone as an Aryl Hydrocarbon Receptor Antagonist in Human Breast Cancer Cells by Jeong Eun Lee and Stephen Safe, 2000. *Toxicological Sciences*, **58**, 235-242. Copyright 2000 by the Society of Toxicology and Involvement of a Post-Transcriptional Mechanism in the Inhibition of CYP1A1 Expression by Resveratrol in Breast Cancer Cells by Jeong Eun Lee and Stephen Safe, 2001. *Biochemical Pharmacology*, **62**, 1113-1124. Copyright 2001 by Elsevier Science Inc.

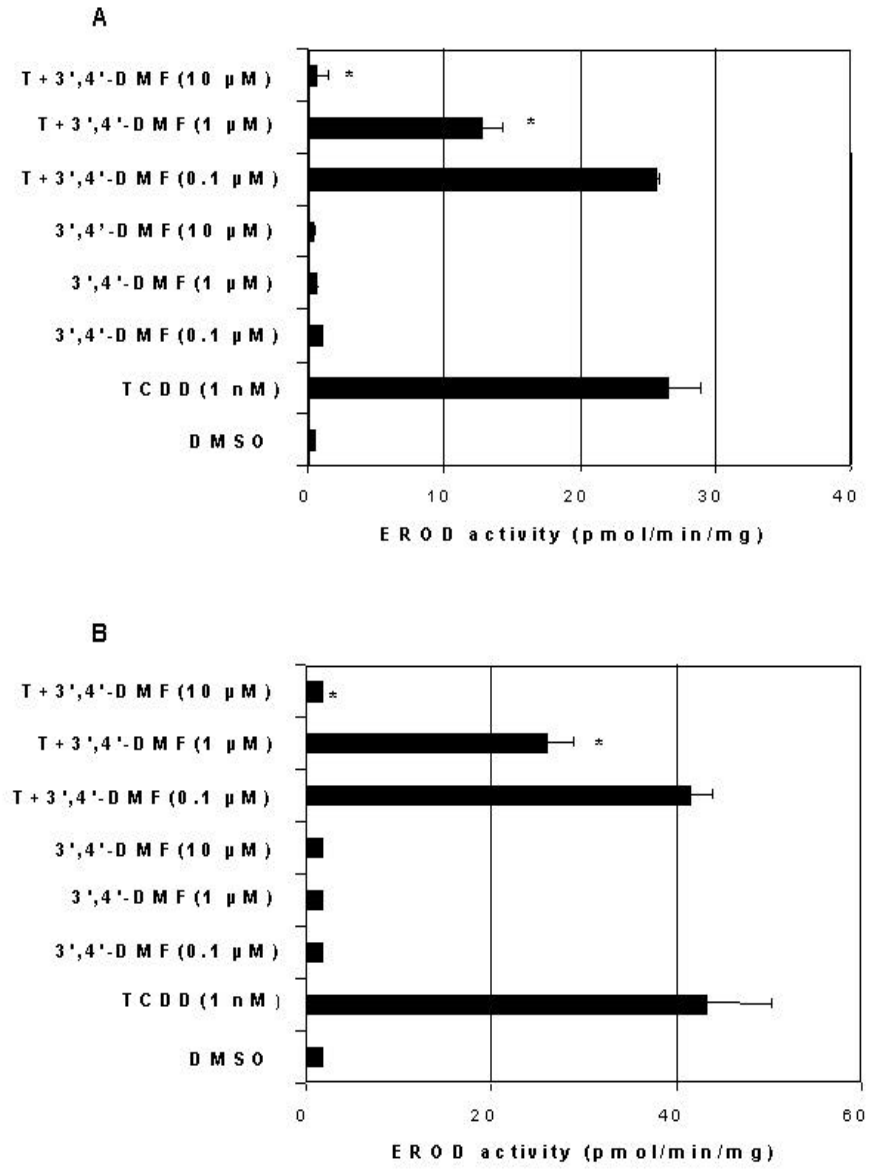


FIG. 16. **Induction of EROD activity by TCDD and inhibition by 3',4'-DMF.** MCF-7 (A) or T47D (B) cells were treated with 1 nM TCDD, 0.1 - 10 μM 3',4'-DMF alone, or 1 nM TCDD plus 3',4'-DMF (0.1 - 10 μM), and EROD activity was determined. TCDD alone significantly ($p < 0.05$) induced EROD, and 1.0 and 10 μM 3',4'-DMF significantly ($p < 0.05$) inhibited this induced response in both cell lines. The results illustrated in Figures 16 through 19 are means ± SE for three replicate experiments for each treatment group.

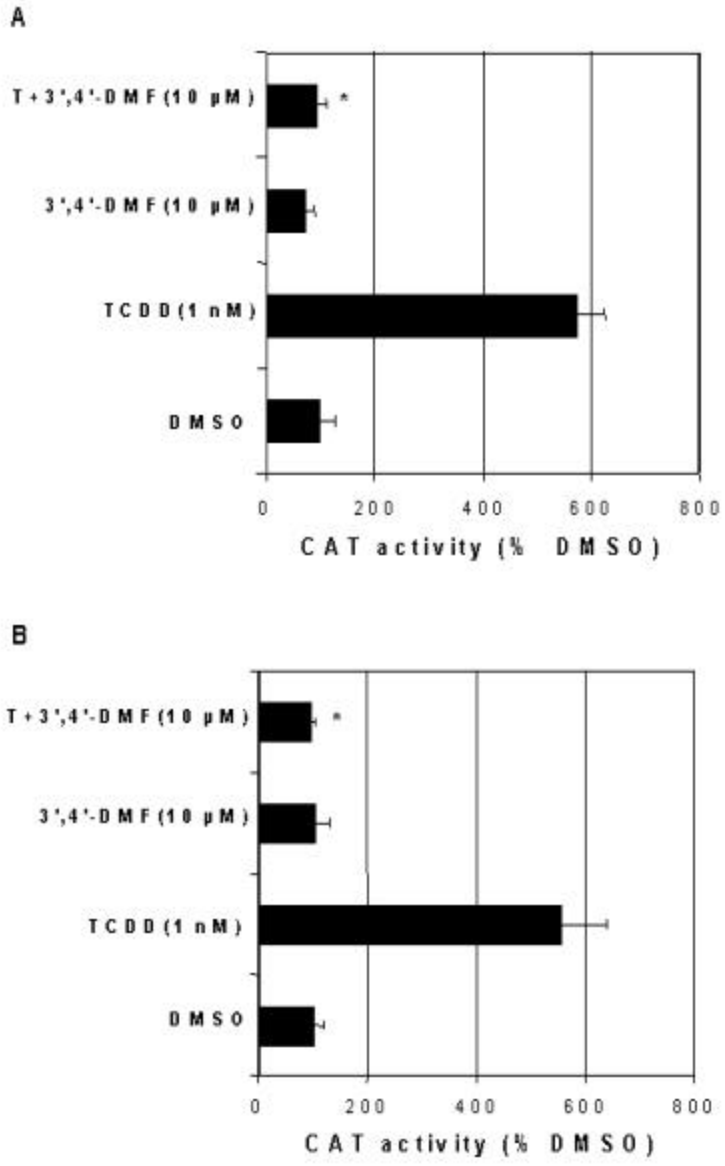


FIG. 17. **Induction of CAT activity by TCDD and inhibition by 3',4'-DMF.** MCF-7 (A) or T47D (B) cells were transiently transfected with pRNH11c, treated with TCDD, 3',4' DMF or their combination, and CAT activity was determined. TCDD alone significantly ($p < 0.05$) induced CAT activity, and cotreatment with 10 μM 3',4'-DMF significantly ($p < 0.05$) inhibited induction of CAT activity by TCDD in both cell lines.

Effect of 3’,4’-DMF on cell proliferation assay

Therefore, we initially investigated the growth inhibitory effects of 3',4'-DMF alone or in combination with E2 in MCF-7 and T47D breast cancer cells. 3',4'-DMF alone significantly induced T47D cell proliferation (0.1 and 1.0 μM) and appeared to exhibit weak estrogen or growth-stimulatory activity in both cell lines; however, this response was not observed at the highest concentration (10 μM) (Fig. 18). In cells treated with 3',4'-DMF plus E2, proliferation was not enhanced by 3',4'-DMF, and 10 μM 3',4'-DMF inhibited E2-induced proliferation of both MCF-7 and T47D cells. Previous studies have also reported growth inhibitory effects of 3'-methoxy-substituted flavones (215), and this may be related to their inhibition of constitutive and hormone-induced kinase activities (193). 3',4'-DMF did not reverse the inhibition of E2-induced proliferation by TCDD (data not shown) and this is probably related to the dose-dependent stimulatory and inhibitory effects of this compound.

Effect of 3’,4’-DMF on E2-inducible CAT assay

Therefore, we also investigated the AhR antagonist activity of 3',4'-DMF in MCF-7 cells transfected with the E2-responsive pCKB construct and treated with E2, TCDD or their combination. The results (Fig. 19) show that E2 induced CAT activity in MCF-7 cells transfected with pCKB, whereas 10 μM 3',4'-DMF or 10 nM TCDD alone were inactive. TCDD, but not 3',4'-DMF, significantly inhibited E2-induced activity, and 3',4'-DMF reversed the inhibition of E2-induced activity by TCDD which is consistent with an AhR antagonist effect by 3',4'-DMF. E2-induced activation of pCKB is not inhibited by AhR agonists in T47D cells (410), and therefore, the AhR antagonist activity of 3',4'-DMF was not determined for this response in T47D cells.

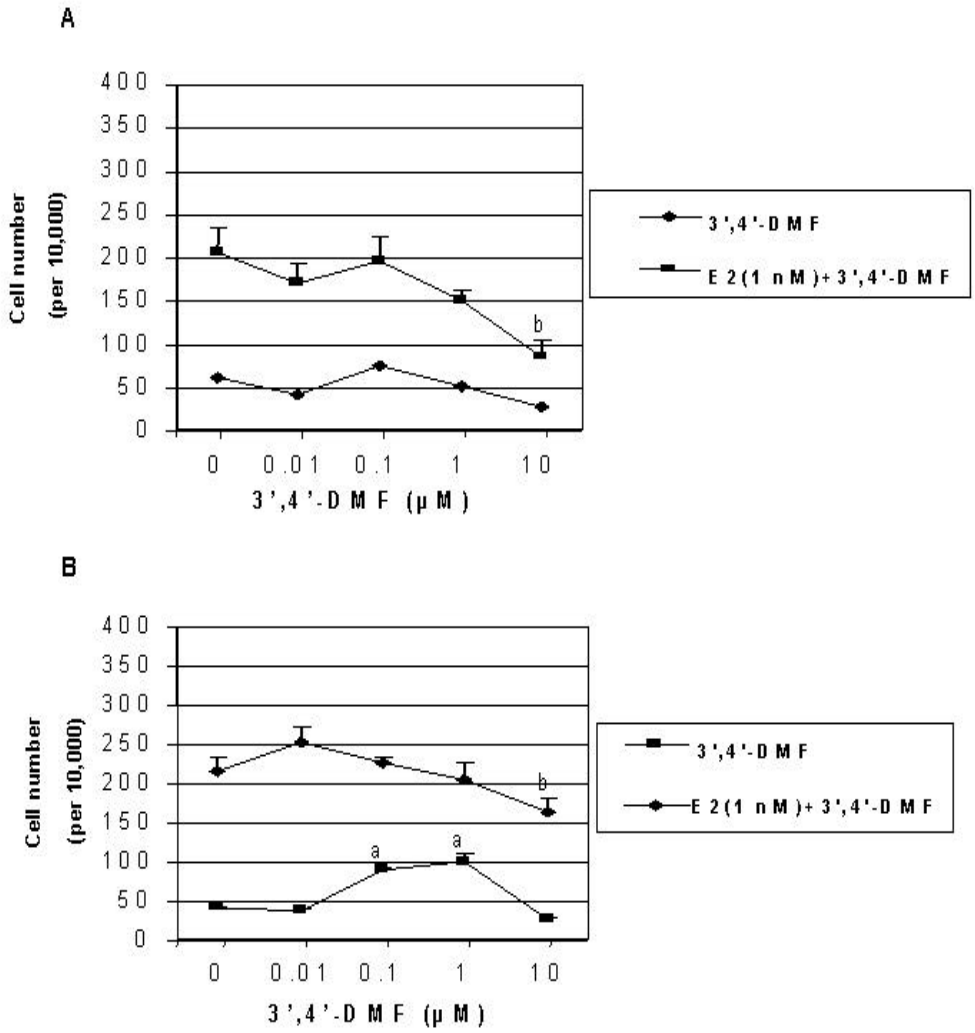


FIG. 18. **Effects of 3',4'-DMF on growth of breast cancer cells.** MCF-7 (A) or T47D (B) cells were treated with different concentrations of 3',4'-DMF alone or in combination with 1 nM E2. Significant ($p < 0.05$)[a] induction of T47D cell growth was observed only at concentrations of 0.1 and 1.0 μM 3',4'-DMF and significant ($p < 0.05$)[b] inhibition of E2-induced growth of both cell lines was observed only at the highest concentration (10 μM) of 3',4'-DMF.

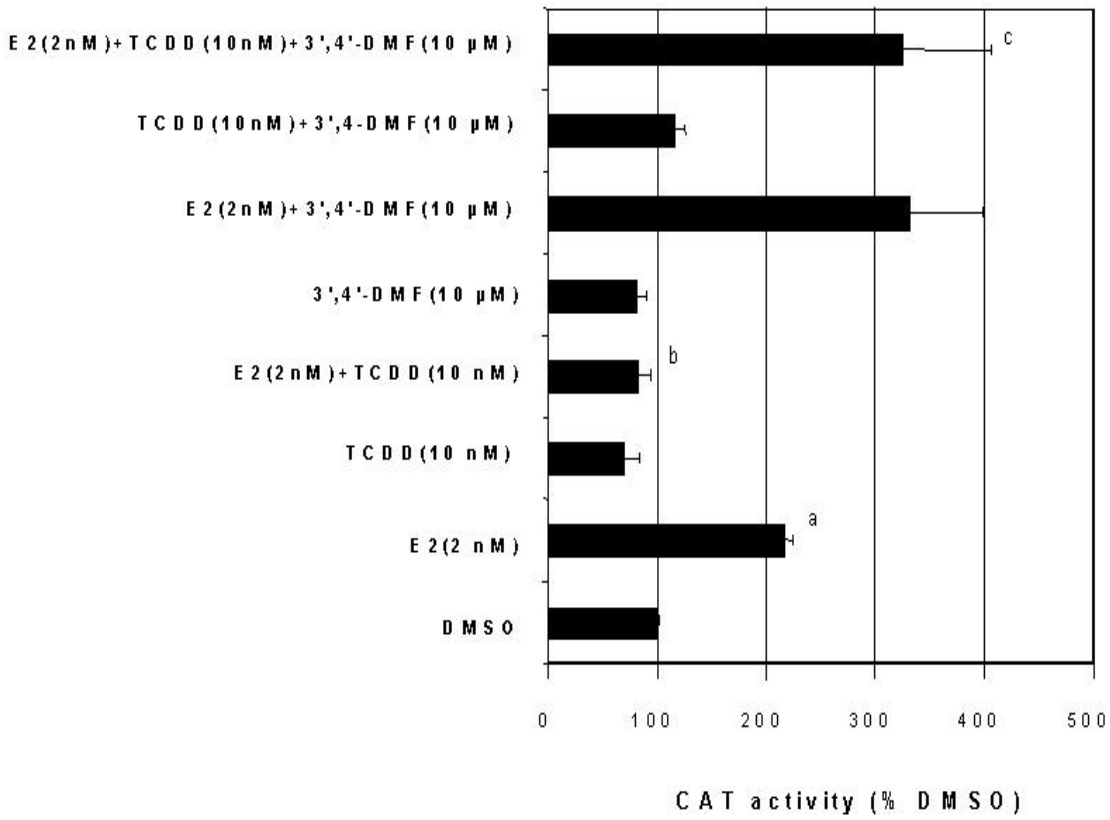


FIG. 19. **Interactive effects of 3',4'-DMF and TCDD on the induction of pCKB by E2.** MCF-7 cells were transfected with pCKB, treated with E2, TCDD, 3',4'-DMF, or their combinations, and CAT activity was determined. E2 significantly induced CAT activity ($p < 0.05$); this response was inhibited by TCDD and the inhibitory response was reversed after cotreatment with 3',4'-DMF.

Effect of 3',4'-DMF on AhR transformation

Thus, 3',4'-DMF blocks TCDD-induced CYP1A1 and inhibitory AhR-ER interactions in breast cancer cells, and the results in Figure 20 illustrate the effects of TCDD, 3',4'-DMF and their combination on the formation of a nuclear AhR complex in MCF-7 and T47D cells. Nuclear extracts from MCF-7 or T47D cells treated with DMSO gave weak to non-detectable binding to [^{32}P]DRE in a gel mobility shift assay. In contrast, an intense band was observed in extracts from cells treated with TCDD and this band was competitively decreased after competition with a 100-fold excess unlabeled wild-type DRE but was unaffected by competition with a mutant DRE oligonucleotide. In contrast, incubation of [^{32}P]DRE with nuclear extracts from MCF-7 or T47D cells treated with 5 nM 3',4'-DMF alone or in combination with TCDD gave minimal to non-detectable retarded bands demonstrating that 3',4'-DMF blocked TCDD-induced formation of the nuclear AhR complex in breast cancer cell lines. Previous studies show that TCDD induced transformation and DRE binding of rat hepatic cytosolic AhR as illustrated in Figure 21. In contrast, 0.5 - 50 μM 3',4'-DMF did not transform the rat cytosolic AhR; however, in cytosols cotreated with TCDD plus 3',4'-DMF, there was significant inhibition of TCDD-induced transformation. These results suggest the 3',4'-DMF competitively binds the cytosolic AhR complex and blocks formation of the transformed nuclear AhR complex, and these results are consistent with previous reports on other 3'-methoxy-substituted flavones (173,180,181,215).

Effects of resveratrol on CYP1A1 gene expression and EROD activity

Results summarized in Figure 22 show that 1 nM TCDD alone induced CYP1A1 mRNA levels (3.5-fold) and CYP1A1-dependent EROD activity in T47D and MCF-7 breast cancer cells, whereas concentrations as high as 10 nM resveratrol were inactive

A

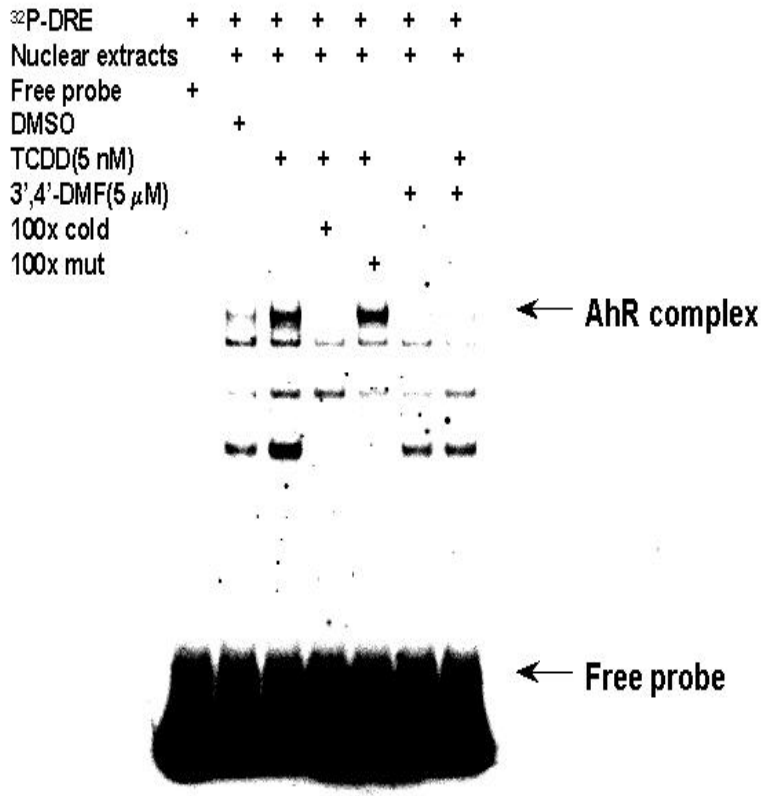


FIG. 20. **Effects of 3',4'-DMF on formation of the nuclear AhR complex.** MCF-7 (A) or T47D (B) breast cancer cells were treated with DMSO, TCDD, 3',4'-DMF or TCDD plus3',4'-DMF, and nuclear extracts were isolated and analyzed by gel mobility shift assays. TCDD induced a specifically-bound complex (see arrow) in both cell lines, whereas nuclear extracts from cells treated with 3',4'-DMF alone or in combination with TCDD gave a minimal to nondetectable bound complex. The mobility of the specifically-bound band was comparable to that observed using *in vitro* translated AhR/Arnt incubated with [^{32}P]DRE (data not shown).

B

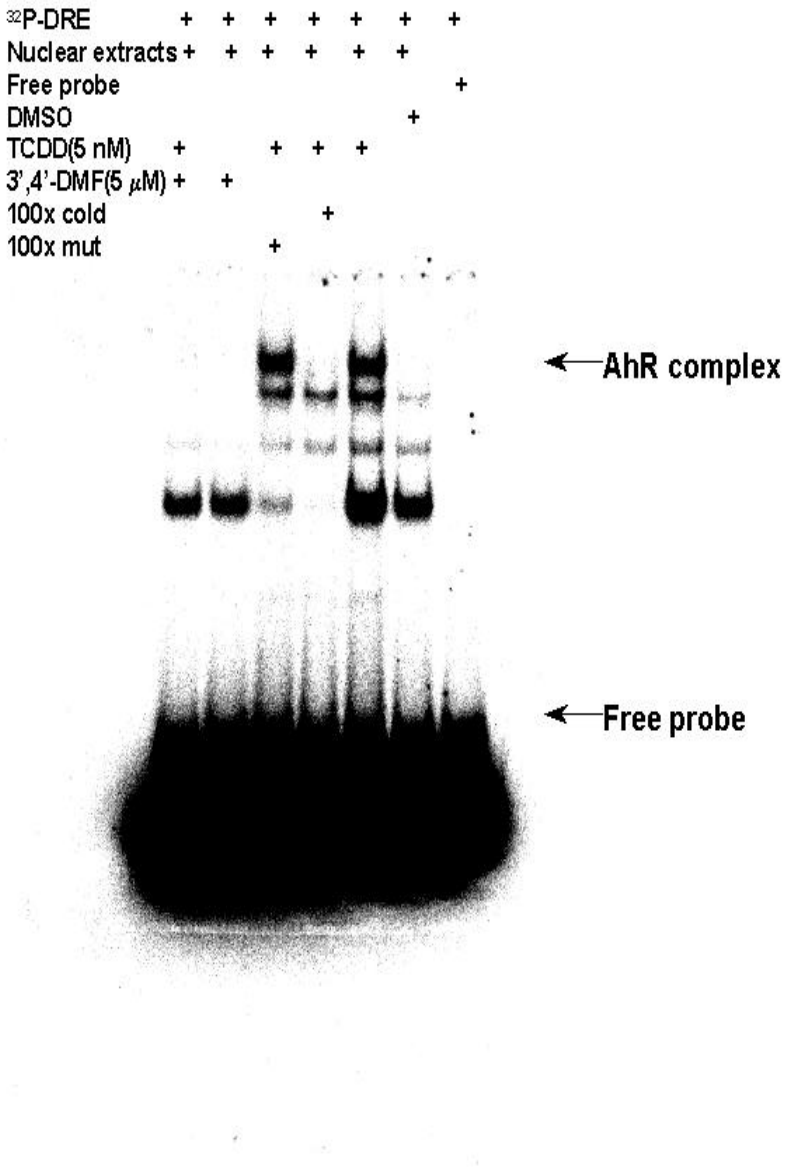


Fig. 20. Continued.

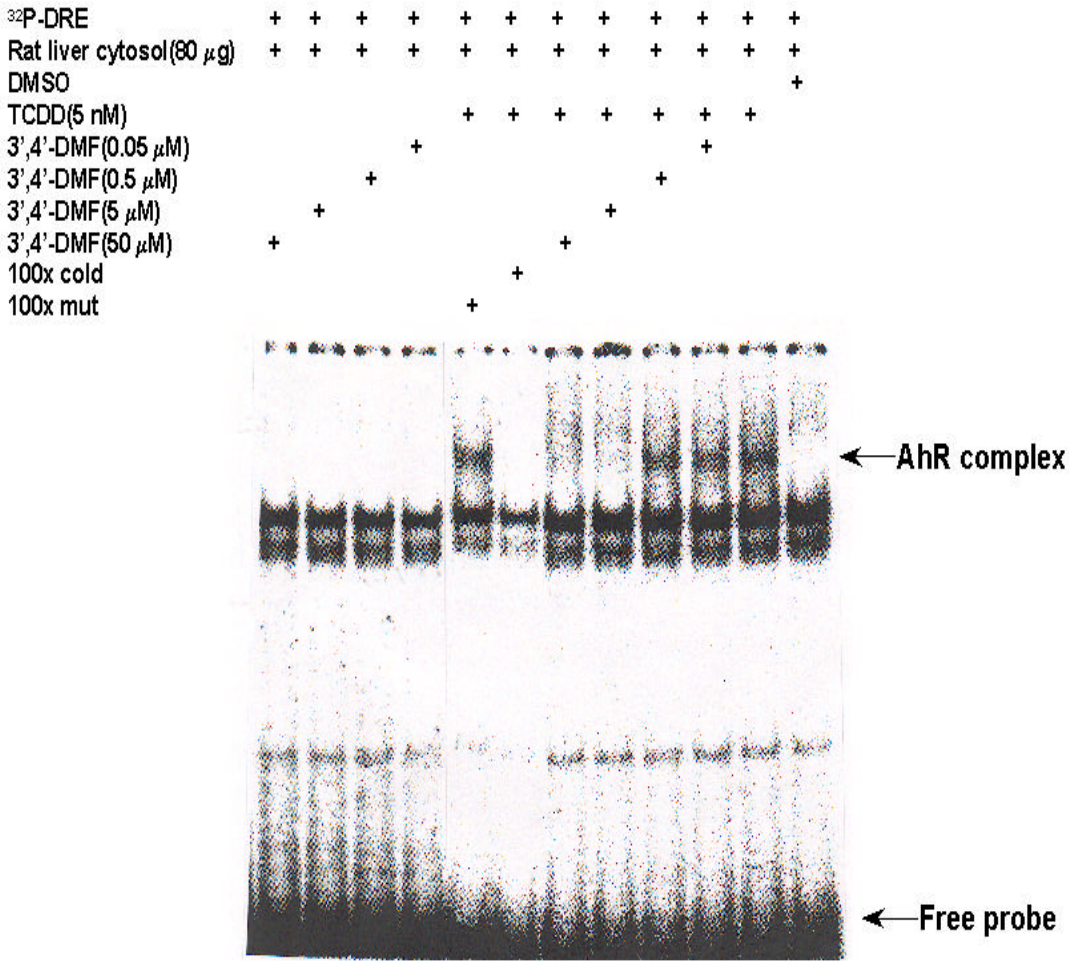


FIG. 21. **Transformation of rat hepatic cytosolic AhR.** Rat hepatic cytosol was Treated with 5 nM TCDD, 3',4'-DMF (0.05 - 50 μM) or their combination and analyzed by gel mobility shift assays. TCDD but not 3',4'-DMF transformed the AhR complex and coincubation of TCDD with 3',4'-DMF resulted in inhibition of TCDD-induced transformation.

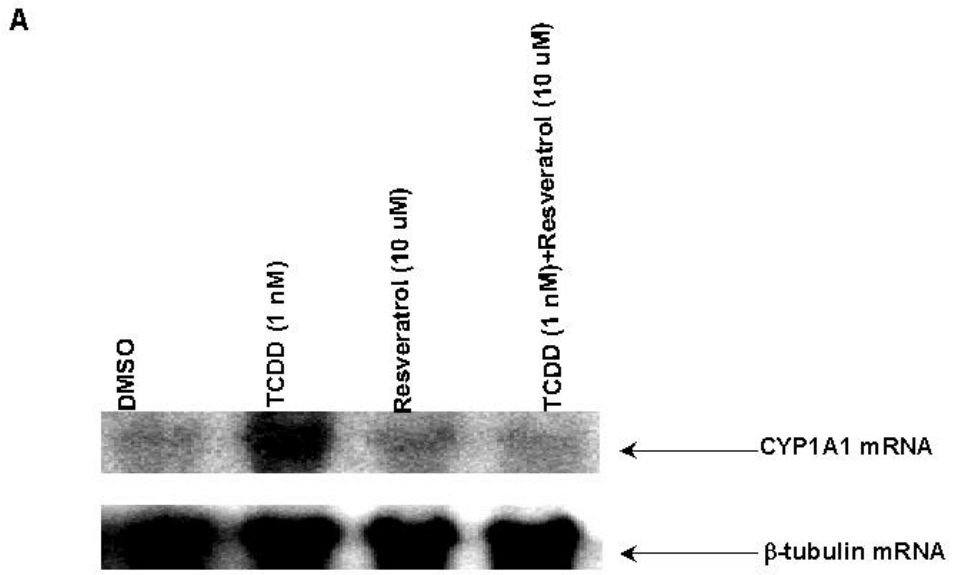


FIG. 22. **Effects of resveratrol on CYP1A1 gene expression in breast cancer cells.** (A) CYP1A1 mRNA levels. T47D cells were treated with solvent control (DMSO), 1 nM TCDD, 10 μM resveratrol and TCDD plus resveratrol (1 nM and 10 μM, respectively), and after 6 hr, mRNA was analyzed by Northern blot analysis as described in the Materials and Methods. Relative CYP1A1 mRNA levels were: DMSO, 1.0 ± 0.08; TCDD, 3.53 ± 0.53; reservatrol, 1.1 ± 0.16, and TCDD plus reservatrol, 0.99 ± 0.1. Results are expressed as means $\pm$ SE for three separate experiments. TCDD significantly ($p < 0.05$) induced CYP1A1 gene expression, and this induced response was significantly ($p < 0.05$) inhibited my resveratrol.

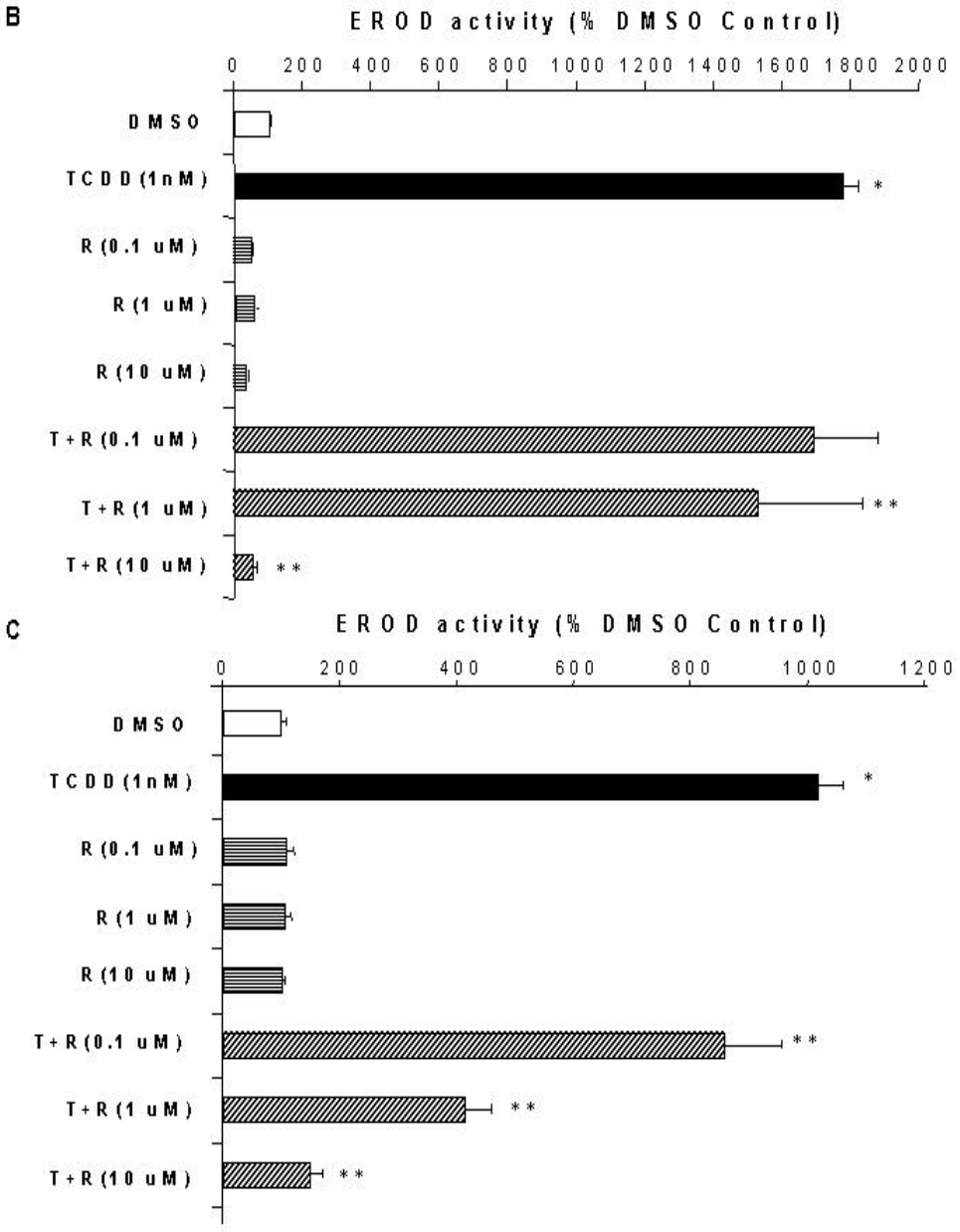


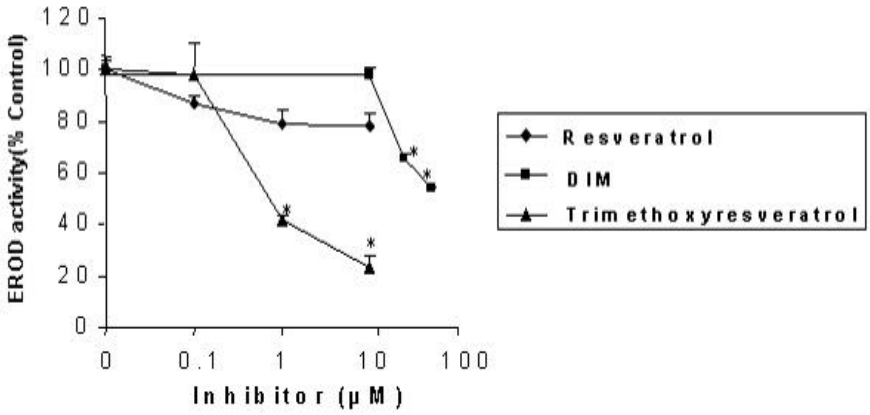
FIG. 22. Continued. EROD activity in T47D (B) or MCF-7 (C) cells. Cells were treated with DMSO, 1 nM TCDD, 0.1 to 10 μM resveratrol, and TCDD (1 nM) plus resveratrol (0.1-10 μM) for 24 hr, and EROD activity was determined as described in the Materials and Methods. TCDD significantly (* $p < 0.05$) induced EROD activity in both cell lines and the induced response was significantly (** $p < 0.05$)inhibited by 1 and 10 μM resveratrol in T47D and 10 μM resveratrol in MCF-7 cells.

as inducers of CYP1A1 mRNA or EROD activity. Induction of CYP1A1 mRNA levels in T47D cells by 1 nM TCDD was blocked after co-administration with 10 μM resveratrol. Moreover, in MCF-7 or T47D cells cotreated with 1 nM TCDD plus 0.1 to 10 nM resveratrol, there was a concentration-dependent decrease in EROD activity and these results were consistent with previous reports on interactions of these compounds with CYP1A1 in various cell lines (266,267,269). Incubation of resveratrol (0.1 - 50 μM) with TCDD-induced microsomes from MCF-7 and T47D cells only slightly decreased enzyme activity (≤ 20%) at the 10 μM concentration and significantly decreased activity was observed at higher concentrations (Fig. 23). Incubation with DIM decreased enzyme activity as previously reported (234), and inhibition was also observed for trimethoxyresveratrol. Thus, the inhibitory effect of 10 μM resveratrol was not related to direct interactions of this compound with CYP1A1 protein reported that microsomal EROD activity and benzo[a]pyrene (BaP) hydroxylation was inhibited after incubation with 10 μM resveratrol (266,267), whereas in another study, the inhibitory effects were similar to those reported in Figure 23 (268). The reason for differences between studies is unclear since the positive inhibitory control (DIM) blocked EROD activity, and we also observed that the trimethoxy derivative of resveratrol inhibited this response.

Interactions of resveratrol with the AhR

Competitive binding of resveratrol to the AhR was not observed using sucrose density gradients (269), whereas in another report, binding was observed in T47D cell whole cell assays (267). These differences were also reflected in results of gel mobility shift assays of nuclear extracts from cells treated with TCDD, resveratrol and their combination (266,267,269). Therefore, these latter studies were repeated in both MCF-7 and T47D cells, and the results are summarized in Figure 24.

A



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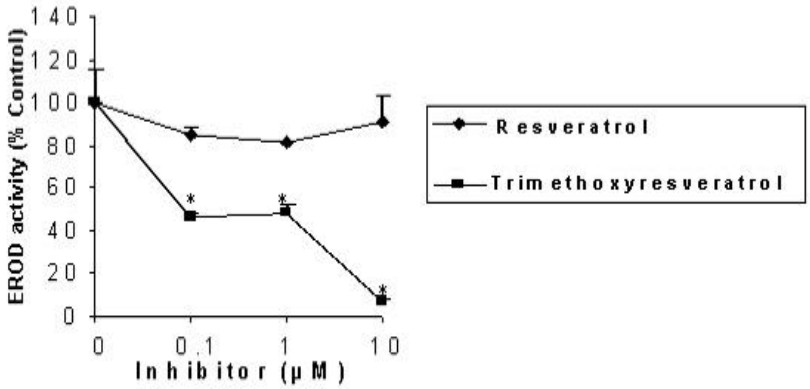


FIG. 23. **Interactions of resveratrol with CYP1A1.** Microsomes from T47D (A) and MCF7 (B) cells were incubated with resveratrol (0.1 - 50 μM), trimethoxyresveratrol (0.1 – 10 μM) or DIM (0.1 - 10 μM) for 10 min, and EROD activity was determined as described in the Materials and Methods. Only minimal (< 22%) inhibition was observed for resveratrol (≤ 10 μM), whereas both trimethoxyresveratrol and DIM inhibited EROD activity. Results are expressed as means ± SE for three separate determinations for each treatment group.

A

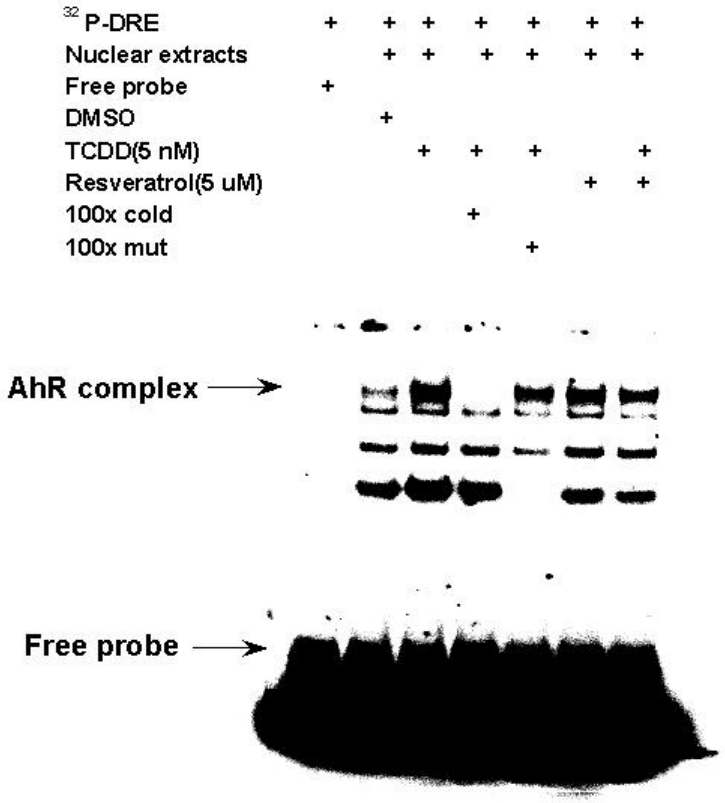


FIG. 24. **Resveratrol-induced formation of the nuclear or transformed AhR complex.** T47D (A) or MCF-7 (B) cells were treated with 5 nM TCDD, 5 μM resveratrol or their combination; nuclear extracts were obtained and analyzed by gel mobility shift assays as described in the Materials and Methods. A specifically-bound AhR-[^{32}P]DRE complex was detected in all treatment groups.

B

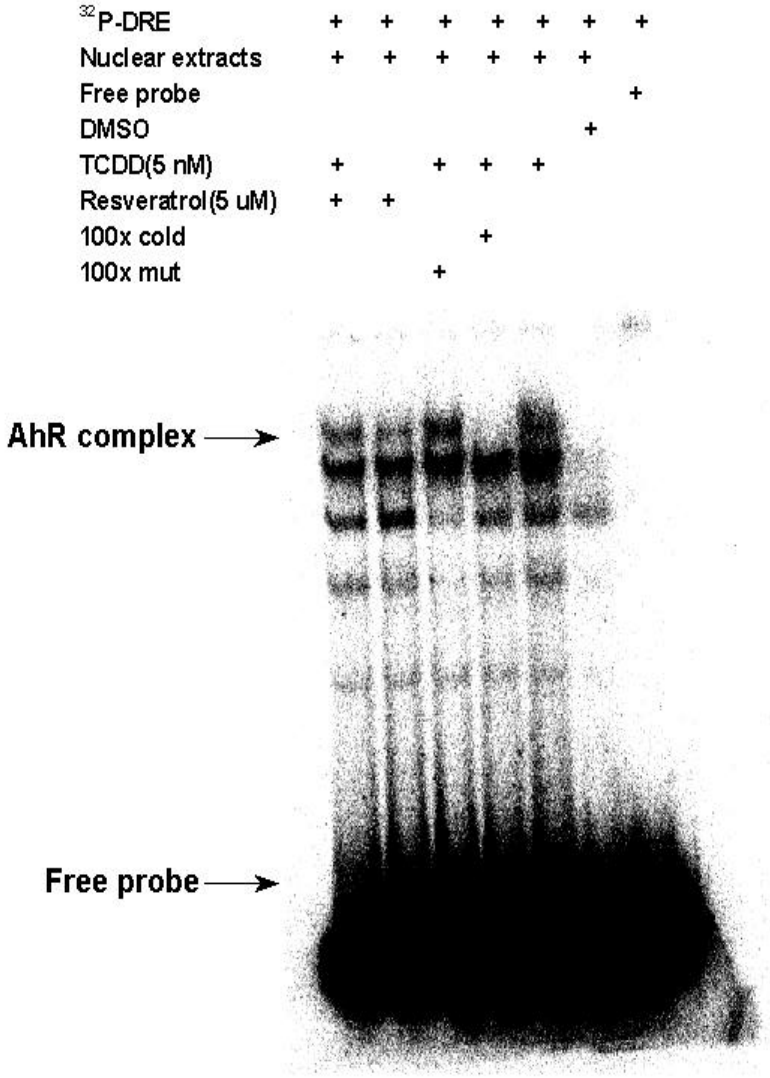


FIG. 24. Continued.

C

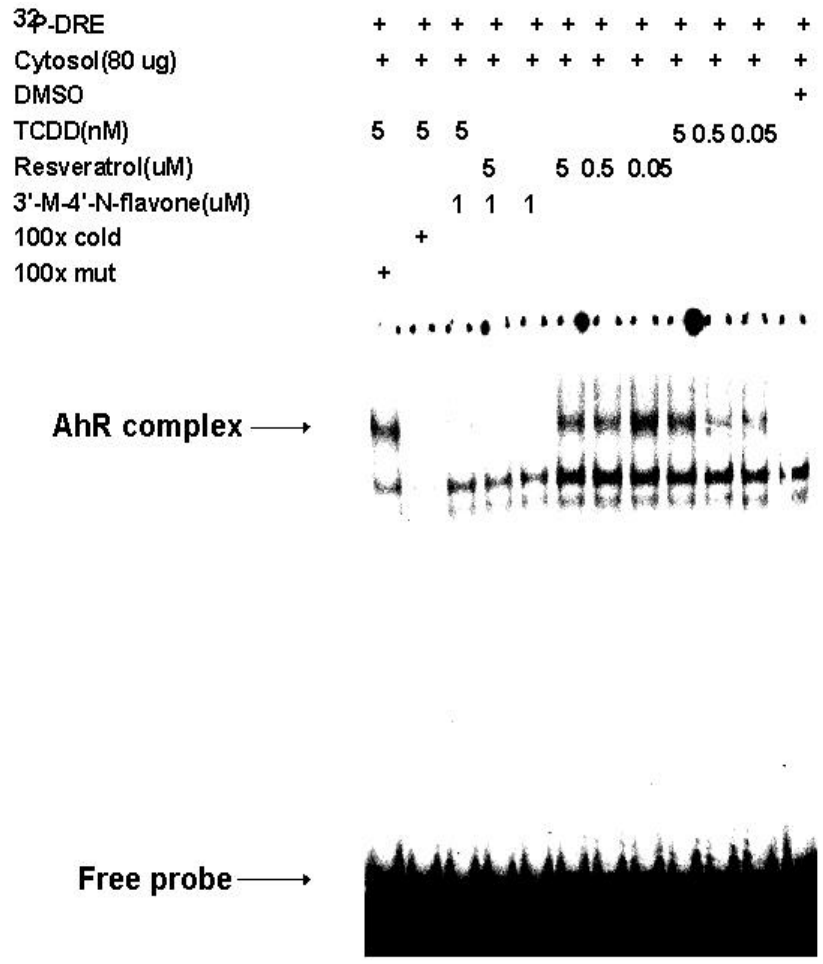


FIG. 24. Continued. (C) Transformation of the rat hepatic AhR. Rat hepatic cytosol was incubated with TCDD, resveratrol, the AhR antagonist 3'-methoxy-4'-nitroflavone or their combinations and the transformed complex was analyzed by gel mobility shift assay as described in the Materials and Methods. TCDD (lanes 9 - 11) and resveratrol (lanes 6 - 8) induced formation of a specifically-bound AhR complex, whereas 3'-methoxy-4'-nitroflavone blocked resveratrol-/ TCDD-induced complex formation but this compound alone did not induce transformation (lanes 3 - 5). This pattern of transformation was observed in duplicate experiments.

Nuclear extracts from MCF-7 and T47D cells treated with 1 nM TCDD were incubated with [^{32}P]DRE and analyzed by gel mobility shift assays to give a specifically-bound retarded band; intensity of this band was decreased by competition with excess unlabeled DRE but not mutant DRE oligonucleotides. Nuclear extracts from cells treated with 5 μM resveratrol alone or in combination with 1 nM TCDD also formed retarded bands; these results were consistent with previous studies in T47D cells (267), but contrasted to results in HepG2 cells where resveratrol did not induce formation of a nuclear AhR complex and blocked formation of a TCDD- or BaP-induced nuclear AhR complex (266,267). The specificity of the effects of resveratrol on transformation of the AhR complex was further investigated using rat liver cytosol (Fig. 24C) which is readily transformed (dose-dependent) by TCDD to give a retarded band (lanes 9 - 11), whereas the solvent (DMSO) did not induce transformation (lane 12). The specificity of TCDD-induced transformation was confirmed by the following; competition with unlabeled wild-type DRE (lane 2), but not mutant DRE (lane 1), decreased intensity of the retarded band and the AhR antagonist 3'-methoxy-4'-nitroflavone blocked TCDD-induced transformation (lane 3) as previously described (173,181). The effects observed for resveratrol show that there was a concentration-independent transformation of the receptor using 0.05 to 50 μM resveratrol (lanes 6 - 8) and coincubation of resveratrol with TCDD did not markedly affect intensities of the retarded bands (data not shown). In contrast, 3'-methoxy-4'-nitroflavone blocked transformation of the cytosolic AhR by resveratrol (lane 4). Results obtained for resveratrol suggest that ligand-induced transformation of the cytosolic AhR may not always predict the AhR agonist or antagonist activity of a compound in transcriptional assays, and this has

recently been demonstrated for several other compounds by Seidel and coworkers (411).

The lack of specificity associated with interactions of resveratrol with the AhR in cell culture and in transformation of rat hepatic cytosol (Fig. 24) suggested that the inhibitory effects of resveratrol on CYP1A1 mRNA and EROD activity induced by TCDD may be AhR-independent. Previous studies have shown that AhR antagonists such as 3'-methoxy-4'-nitroflavone and other 3'-methoxysubstituted flavones inhibit formation of the nuclear AhR complex and induction of reporter gene activity by TCDD in cells stably- or transiently transfected with constructs containing functional DRE promoter inserts (173,180,181). Ciolino and Yeh (266) also showed that resveratrol inhibited CAT activity in HepG2 and MCF-7 cells treated with AhR agonists and transiently transfected with a construct containing a rat CYP1A1 gene promoter insert. In contrast, our results (Fig. 25) show that 1 and 10 μM resveratrol alone did not induce CAT activity in T47D or MCF-7 cells transfected with Ah-responsive pRNH11c; TCDD alone induced CAT activity and the induced response was not affected by 1 or 10 μM resveratrol, whereas the AhR antagonist 3'-methoxy-4'-nitroflavone blocked TCDD-induced reporter gene activity as previously described (173,181). Thus, in contrast to 3'-methoxy-4'-nitroflavone, resveratrol did not exhibit AhR antagonist activity for this response.

Effects of resveratrol on CYP1A1 mRNA and protein stability

The apparent conflicting results on the AhR antagonist activity of resveratrol obtained in this and previous studies suggested that other inhibitory mechanisms may also be important. A recent study showed that the steroid hormone DHEA inhibited basal and induced CYP1A1 gene expression in MCF-7 cells but had no effect on DRE-dependent transcription using a construct containing the rat CYP1A1 gene promoter

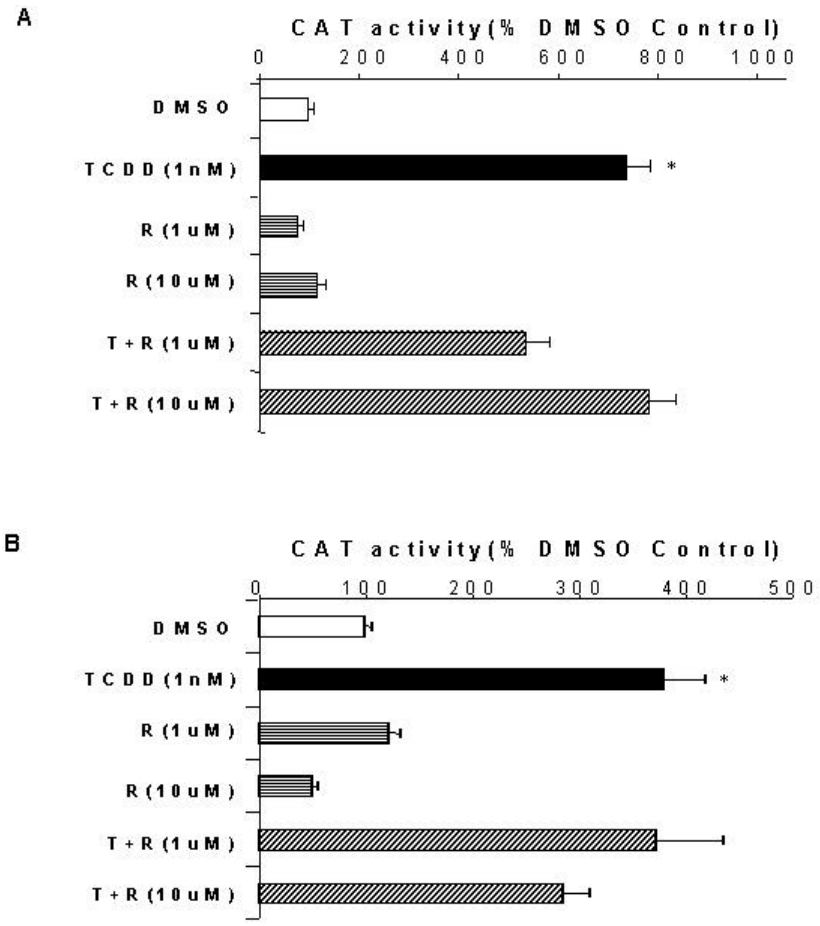


FIG. 25. **AhR agonist/antagonist activity of resveratrol in transfection studies.** T47D (A) or MCF-7 (B) cells were transfected with pRNH11c, treated with TCDD, resveratrol, or their combinations, and CAT activity was determined as described in the Materials and Methods.

C

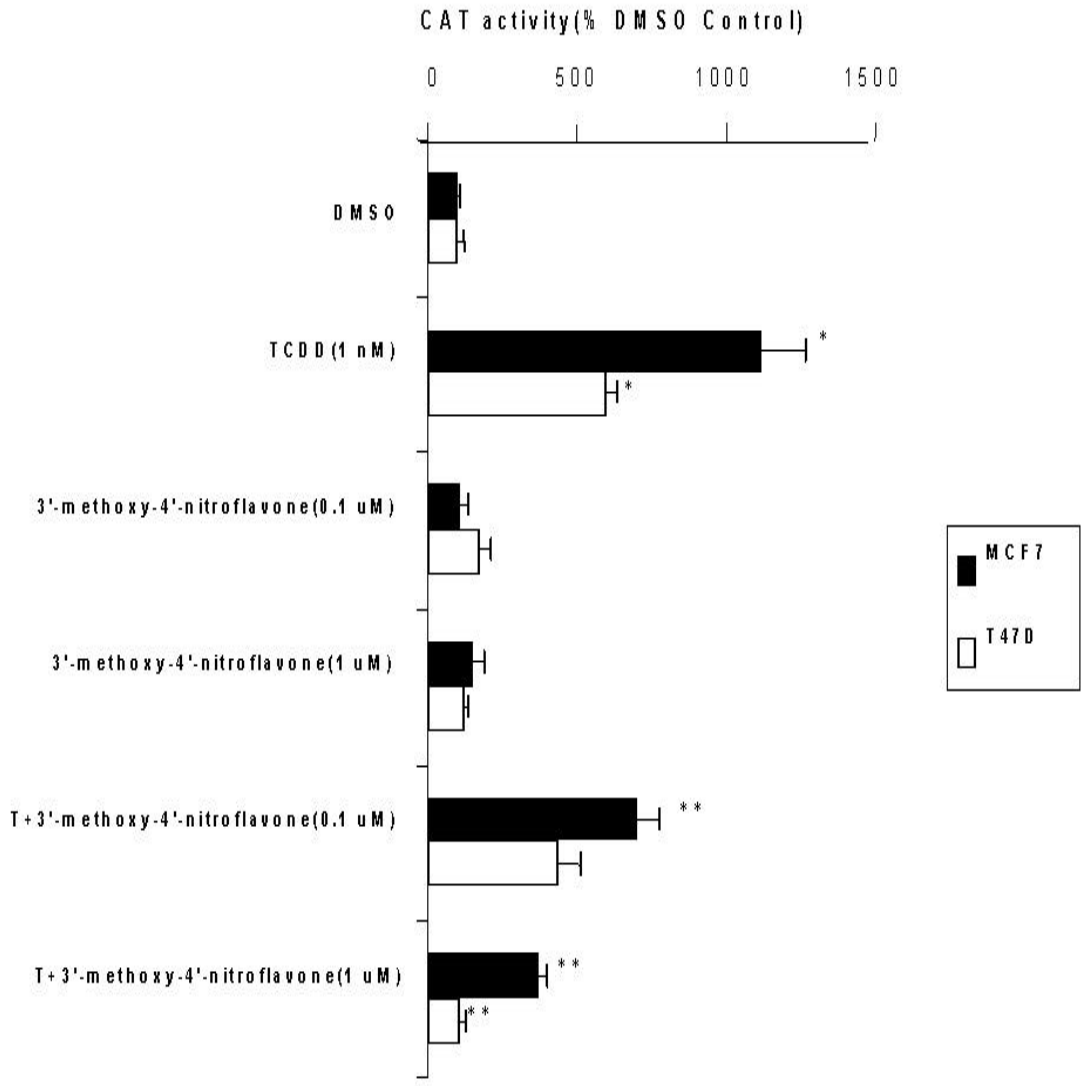


FIG. 25. Continued. In a separate experiment (C), the effects of the AhR antagonist 3'-methoxy-4'-nitroflavone (F) on TCDD-induced CAT activity were also determined. Only TCDD alone significantly (* $p < 0.05$) induced CAT activity and in combination treatments, only 3'-methoxy-4'-nitroflavone significantly (** $p < 0.05$) decreased the TCDD-induced response. Results are expressed as means ± SE for three separate experiments for each treatment group.

insert (145). The inhibitory effect of DHEA was associated with post-transcriptional destabilization of CYP1A1 mRNA and, therefore, the time-dependent effects of DHEA and resveratrol were determined in T47D cells pretreated with TCDD to induce CYP1A1 mRNA levels. Actinomycin D was then added to inhibit further transcription and CYP1A1 mRNA levels (normalized to β-tubulin mRNA) were then determined 0, 2, 6 and 10 hr after addition of actinomycin D. The results (Fig. 26) show that both DHEA and resveratrol significantly increased the rate of CYP1A1 mRNA degradation suggesting that, like DHEA, resveratrol also inhibits CYP1A1 by post-transcriptional mechanisms. Moreover, like resveratrol, DHEA also inhibited TCDD-induced EROD activity in T47D cells (data not shown). It is also possible that resveratrol could induce proteosome-dependent degradation of CYP1A1 protein, and the effects of resveratrol, the proteosome inhibitor MG132 and their combination on CYP1A1-dependent EROD activity were determined in T47D cells (Fig. 27). Eighteen hours after treatment with 1 nM TCDD, the media was changed and cells were further incubated with resveratrol or resveratrol plus MG132 for a further 12 hr. None of the treatments deceased CYP1A1-dependent activity suggesting that the effects of resveratrol on CYP1A1 expression are primarily directed to decreased message stability. These results coupled with the non-specific interactions of resveratrol with the AhR (Fig. 24) indicate that resveratrol may inhibit CYP1A1 gene/protein expression, in part, by AhR-independent pathways.

DRIP150 coactivation of ERα

DRIP150 is a member of the mediator complex of proteins, and this study investigates coactivation of ERα by DRIP150 in ZR-75 cells transfected with $pERE_3$. E2-dependent transactivation in this cell line is minimal in cells transfected with $pERE_3$ alone; however, E2-reponsiveness is observed after cotransfection with minimal

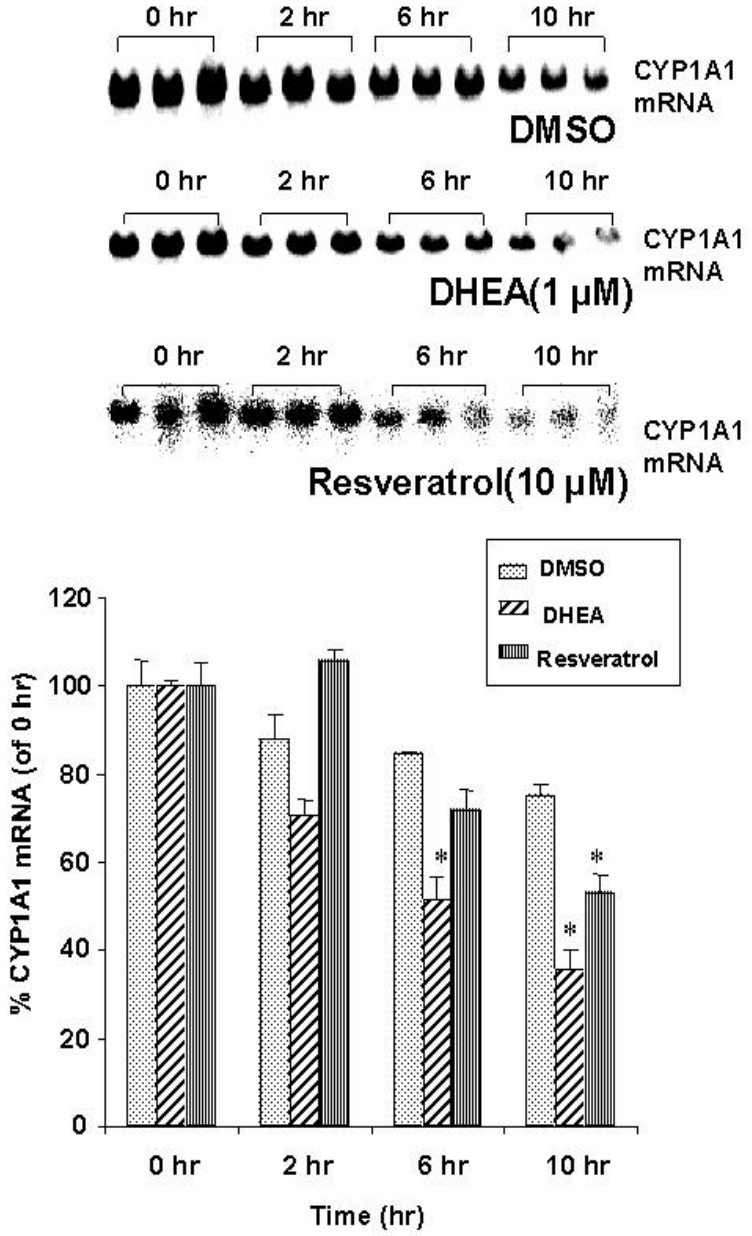


FIG. 26. **CYP1A1 mRNA stability in T47D cells treated with resveratrol and DHEA.** T47D cells were pretreated with 1 nM TCDD for 12 hr; media was changed and cells were treated with 5 μM resveratrol, 1 μM DHEA, or DMSO plus 5 μg/ml actinomycin D. CYP1A1 mRNA levels were determined 2, 6 and 10 hr after treatment as described in the Materials and Methods. CYP1A1 mRNA levels (relative to β-tubulin mRNA) are illustrated for cells treated with DMSO, DHEA or resveratrol, and both DHEA and reservatrol significantly ($p < 0.05$) decreased mRNA stability at the 10 hr time point.

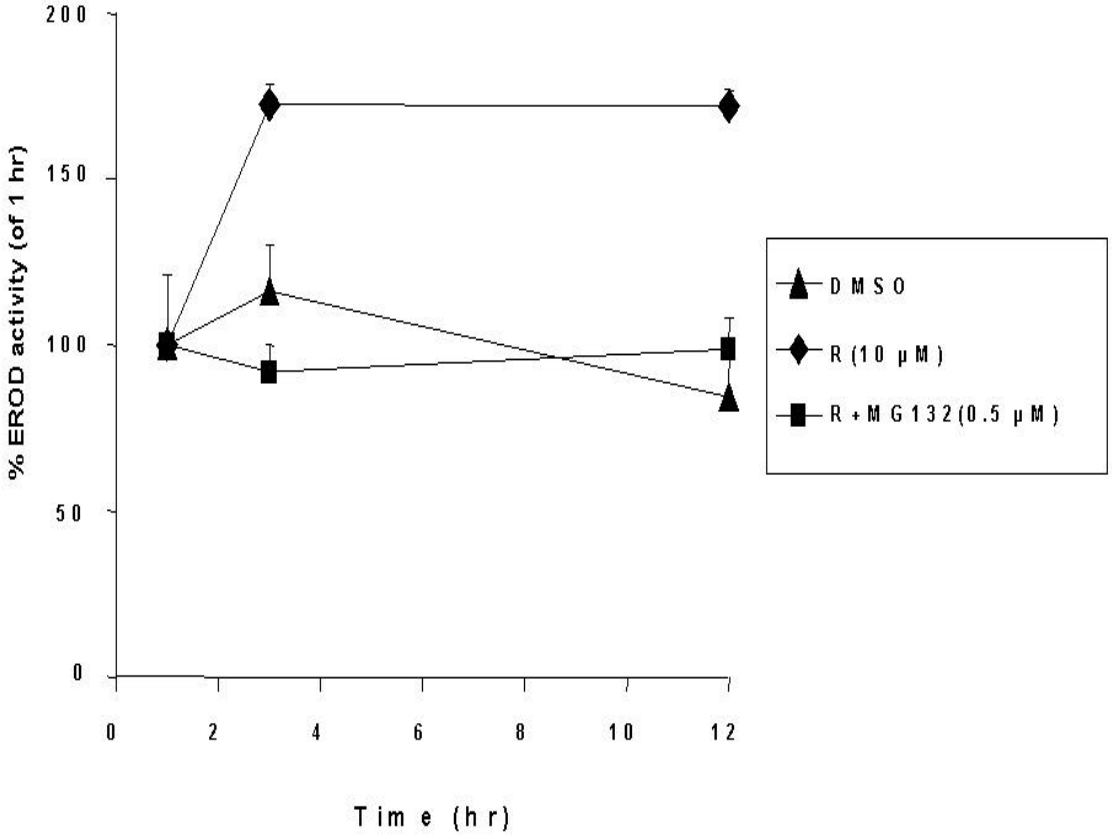


FIG. 27. **Effects of resveratrol on CYP1A1-dependent EROD activity.** T47D cells were treated with 1 nM TCDD for 18 hr; media was changed and cells were then treated with DMSO (control), 10 µM resveratrol, and 10 µM resveratrol plus 0.5 µM MG132 (a proteasome inhibitor). EROD activity was then determined after further incubation for 1, 3 and 12 hr as described in the Material and Methods. The concentration of MG132 was sufficient for inhibition of TCDD-induced proteosome-dependent degradation of the AhR in T47D cells (431). The results showed that resveratrol did not affect protein stability as determined by CYP1A1-dependent EROD activity. Results are expressed as means $\pm$ SEM for three separate experiments for each treatment group.

amounts of ERα expression plasmid. This is due, in part, to overexpression of $pERE_3$ in the transfected cells which results in limiting levels of ERα. The three tandem consensus EREs are inserted upstream from a minimal TATA-luciferase which has lower intrinsic E2-responsiveness compared to constructs containing the human thymidine kinase or frog vitellogenin A2 gene promoters (412). E2 induced luciferase activity in ZR-75 cells transfected with $pERE_3$ and enhancement of this response was variable and dependent on the amount of cotransfected ERα as illustrated in Figure 28A. Maximal coactivation of ERα was observed using 2.5 - 5.0 ng of DRIP150 expression plasmid and the coactivation response was decreased or squelched using higher amounts of DRIP150 plasmid. ZR-75 cells were also transfected with GAL4-luc and pM-ERα which contained full length ERα fused to the DBD of GAL4, and E2 also induced transactivation in this mammalian one-hybrid assay. This response was also enhanced by cotransfection with DRIP150 expression plasmid (Fig. 28B). Coactivation required higher amounts of transfected DRIP150 in this assay, and DRIP150 did not affect hormone-induced transactivation in cells transfected with pM alone which contained the GAL4-DBD but not the ERα insert (data not shown). Figure 28C was similar to that described in Figure 28A and shows that DRIP150 also coactivates ERα-mediated transactivation in ER-negative MDA-MB-231 breast cancer cells transfected with $pERE_3$.

ERα contains two major activation domains and we therefore investigated the coactivation activity of DRIP150 in cells transfected with HE19 (deletion of AF1) and ERα-TAF1 which contains three amino acid mutations in helix 12 (D538N, E542Q and D545N) which inactivates AF2 (413). The results in Figure 28D demonstrate that in ZR-75 cells transfected with $pERE_3$ and HE19, treatment with E2 increased (> 50%)

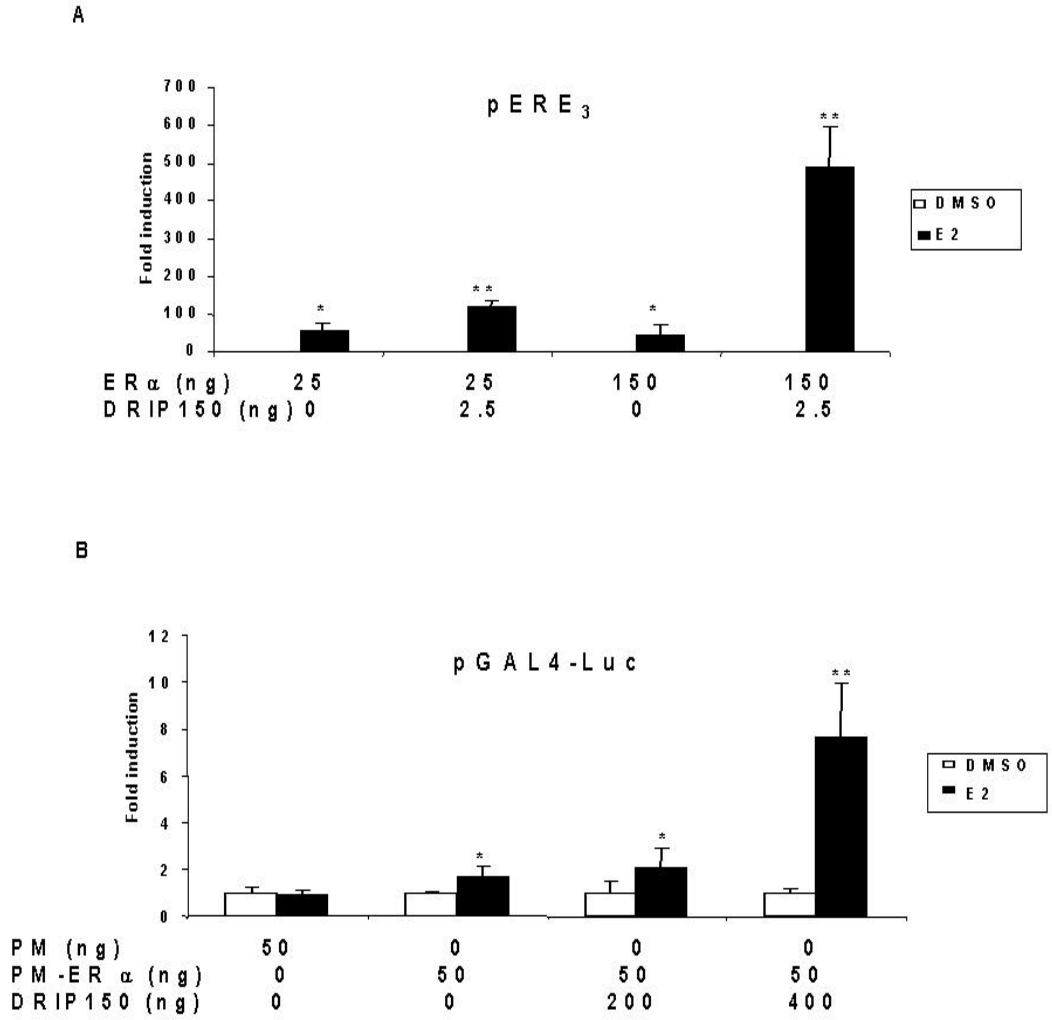
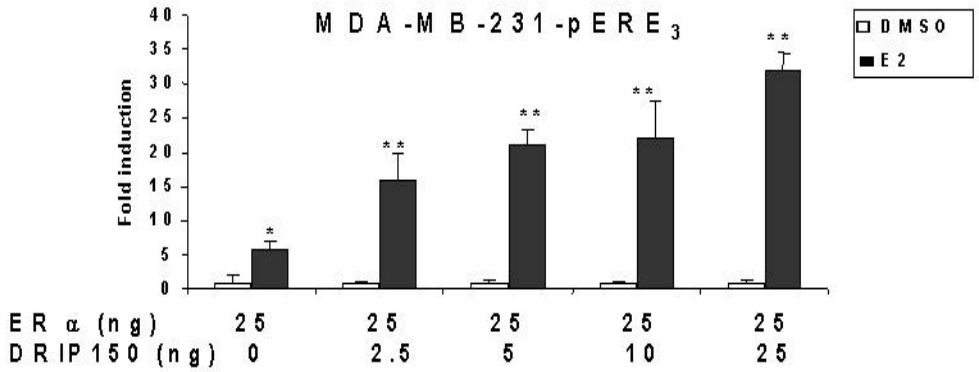


FIG. 28. **Coactivation of wild-type and variant ERα by DRIP150.** (A) Coactivation of ERα. ZR-75 cells were transfected with pERE$_3$, different amounts of ERα expression plasmid (25 and 150 ng), DRIP150 expression plasmid (2.5 ng) treated with DMSO or 10 nM E2 and luciferase activity determined as described in the Materials and Methods. Significant (0 < 0.05) coactivation of E2-induced activity is indicated by an asterisk and results are expressed as means ± SE for at least three separate determinations for each treatment group. Significant coactivation by DRIP150 (or mutants) in this study represents an increase in the fold induction compared to that observed for E2 alone. Hormone responsiveness was not observed in the absence of cotransfected ERα. (B) Coactivation of pM-ERα by DRIP150. ZR-75 cells were transfected with pM (empty vector), pM-ERα (50 ng), or DRIP150 (200 or 400 ng), treated with E2 or DMSO, and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by E2 (*) and coactivation by DRIP150 (**) is indicated.

C



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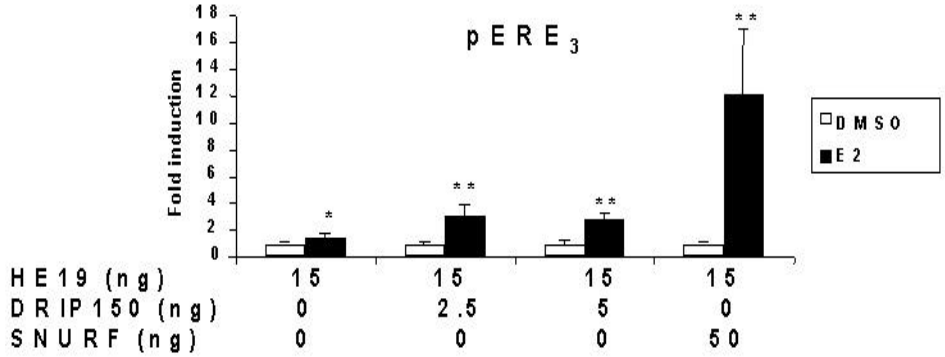

FIG. 28. Continued. (C) Coactivation of ERα in MDA-MB-231. Cells were treated at described in (A) and significant ($p < 0.05$) coactivation by DRIP150 is indicated (**). Coactivation of HE19 (D) and TAF1 (E) by DRIP150. ZR-75 cells were treated with 10 nM E2 or DMSO, transfected with the indicated amounts of plasmids, and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) coactivation is indicated (**). SNURF coactivation of ERα (D) and DRIP150 coactivation of ERα (E) serve as positive controls for these experiments.

E

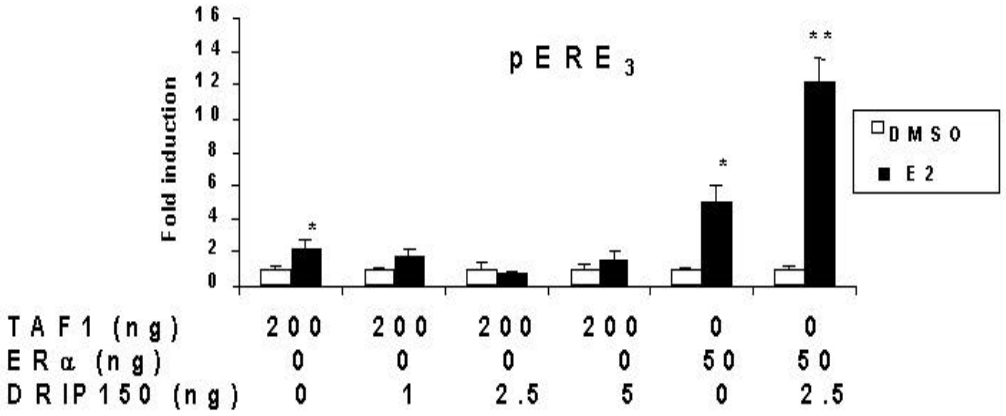

FIG. 28. Continued.

luciferase activity and this response was further enhanced by cotransfection with DRIP150. As a positive control for this experiment, we also observed coactivation of HE19 by the RING finger protein SNURF as previously reported (408). A higher level of coactivation by SNURF was observed and this may be due, in part, to the cooperative coactivation of HE19 by SNURF and TATA binding protein (408). E2 induced luciferase activity in ZR-75 cells transfected with ERα-TAF1; however, this response was not enhanced by DRIP150 (Fig. 28E), whereas in the same experiment, DRIP150 coactivated wild-type ERα (positive control).

These results suggest that DRIP150 primarily coactivates ERα through direct or indirect interactions with the AF2 domain and this was further investigated by competition (squelching) experiments with NR-box and AF1 proteins (AF1-p). The results in Figure 29A demonstrate that increasing amounts of the 2XF6 peptide (387) which contains 2 NR boxes fused to the yeast GAL4-DBD significantly decreased DRIP150 coactivation of ERα in ZR-75 cells. In contrast, transfection with the AF1 protein which contains amino acids 1-182 from ERα did not significantly decrease (or squelch) DRIP150 coactivation of ERα (Fig. 29B), whereas this protein inhibited AF1-dependent activation of GC-rich promoter constructs by ERα/Sp1 (414). These results suggest that coactivation of ERα by DRIP150 is primarily AF2-dependent.

Interactions of ERα and DRIP150

Kang and coworkers reported that in nuclear extracts containing DRIP complex proteins, both wild-type and the ligand binding domain of ERα interacted with DRIP150 in pulldown assays; however, DRIP150-ERα interactions were not observed unless DRIP205 was also expressed (415). Results illustrated in Figure 30A used *in vitro*

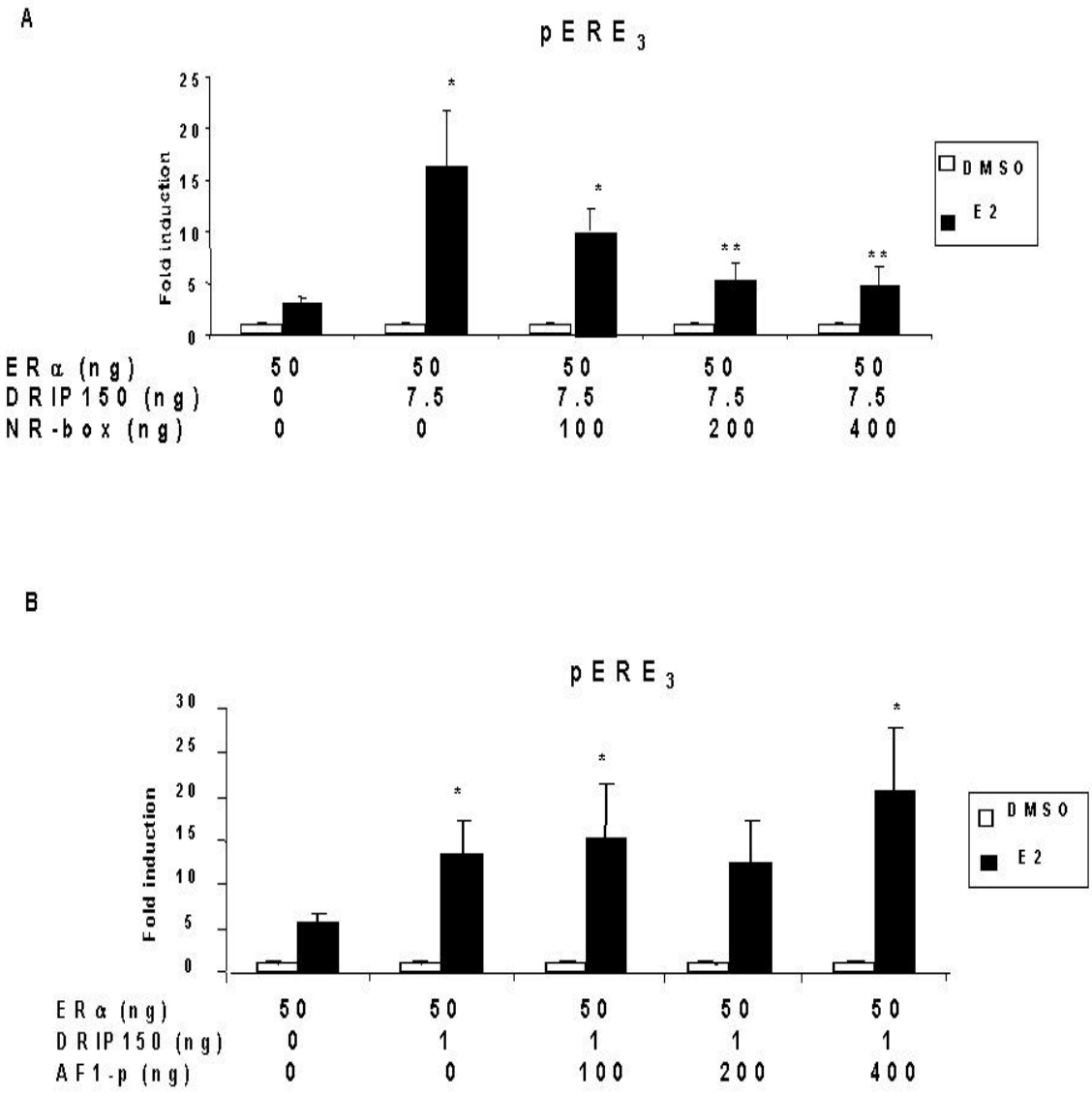

FIG. 29. **Squelching of DRIP150 coactivation of ERα by NR-box (A) and ERα-AF1 (B) peptides.** ZR-75 cells were treated with DMSO or 10 nM E2, transfected with $pERE_3$, DRIP150 (1 or 7.5 ng), ERα expression plasmid (50 ng), and different amounts of NR box or AF1-ERα expression plasmids, and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) coactivation by DRIP150 is indicated (*) and inhibition by ERα-AF or NR-box expression is only indicated (**).

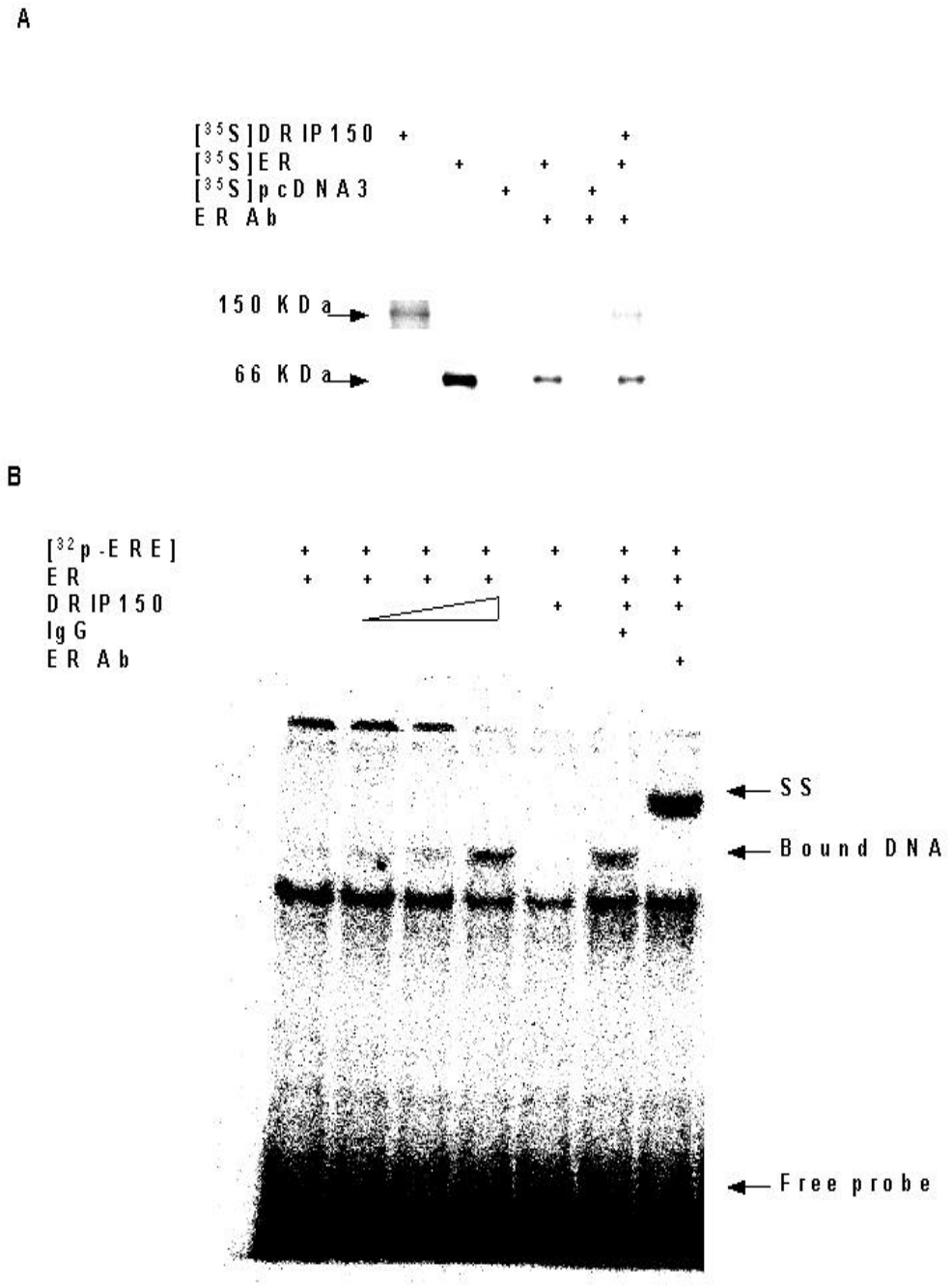

FIG. 30. **DRIP150-ERα interactions.** (A) Coimmunoprecipitation. *In vitro* expression of ^{35}S-labeled DRIP150 or ERα were immunoprecipitated by ERα antibodies as described in the Materials and Methods. [^{32}P]PcDNA3 (empty vector) served as a control. (B) Gel mobility shift assays. [^{32}P]ERE was incubated with ERα, *in vitro* expressed DRIP150 in the presence or absence of IgG (non-specific) or ERα antibodies, and examined in a gel mobility shift assay as described in the Materials and Methods. In a separate experiment, excess unlabeled ERE also decreased intensity of the retarded of the specifically bound retarded band (Bound DNA).

translated radiolabeled [^{35}S]ER and [^{35}S]DRIP150 in coimmunoprecipitation experiments. ERα antibodies coimmunoprecipitate ERα alone and in combination with DRIP150 indicating that both proteins directly interact. Interactions of the *in vitro* translated proteins (unlabeled) were also investigated in gel mobility shift assays using [^{32}P]ERE (Fig. 30B). Incubation of ERα and [^{32}P]ERE gave a retarded band (lane 1) and coincubation with increasing amounts of DRIP150 increased intensity of the retarded band (lanes 2 - 4) but a supershifted ternary complex was not observed. DRIP150 alone did not form a complex with [^{32}P]ERE (lane 5), and the DRIP150-enhanced complex was supershifted by ERα antibodies (lane 7) but not by non-specific IgG (lane 6). Thus, DRIP150 enhanced ER-ERE complex formation, and similar observations have previously been reported for other transcription factors (including ERα) in gel mobility shift assays where two interacting proteins did not form a ternary complex with DNA; however, protein-DNA binding of one protein was enhanced by the other protein (100,416-418).

Coactivation of ERα by mutant DRIP150 constructs

Wild-type DRIP150 contains 1454 amino acids (aa) with two putative NR boxes at 1182-1186 and 73-69. Figure 31A summarizes the effects of wild-type DRIP150 and mutants containing deletions of aa 1454-1145 (DRIP150m1), 1454-789 (DRIP150 m2), and 1454-325 (DRIP150m3) on coactivation of ERα in ZR-75 cells transfected with $pERE_3$. The results show that DRIP150m1 was the only one of these deletion mutants that coactivated ERα, suggesting that the C-terminal NR-box was not required for coactivation, and the N-terminal NR-box was not sufficient for coactivation. The results also indicate that the aa 1144-789 are required for coactivation of ERα on an ERE promoter. The results in Figure 31A were obtained using 2.5 ng of wild-type/mutant

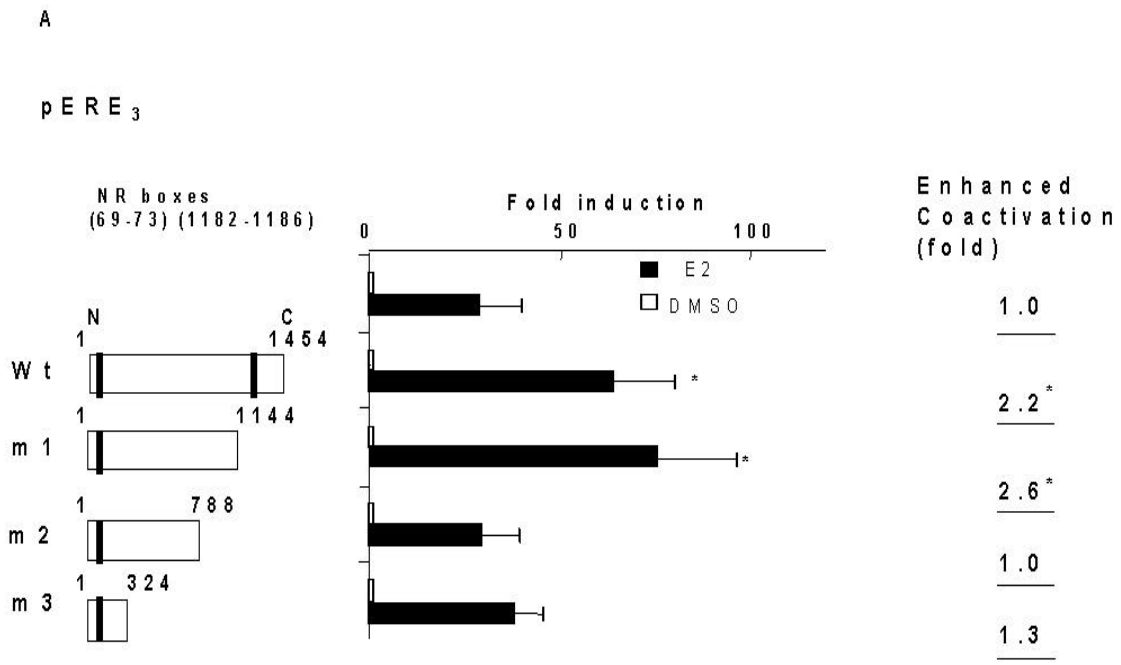

FIG. 31. **Coactivation of ERα by wild-type and variant DRIP150 and their interactions in gel mobility shift assays.** Coactivation of ERα by wild-type DRIP150 and mutants 1 - 3 (A), 4 - 7 (B), and 9 - 12 (C). ZR-75 cells were transfected with $pERE_3$, (50 ng) and wild-type variant DRIP150 expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SE for three separate experiments for each treatment group. All experiments were carried out over a range of DRIP150 (wild-type/variant), and the maximal enhanced coactivation (fold) is reported.

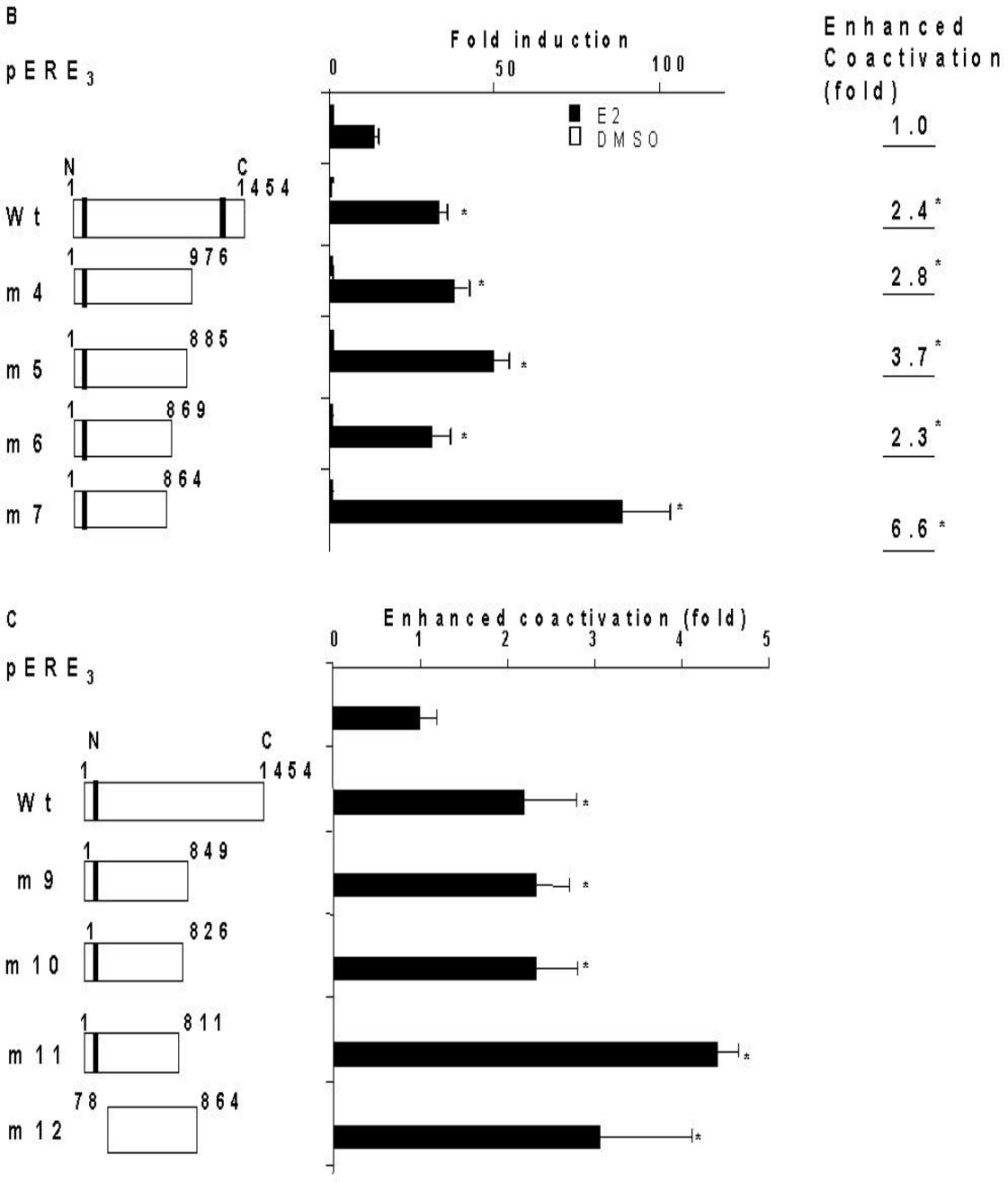

FIG. 31. Continued. Results in Figure 31C were carried out in several separate experiments and combined to show the enhanced coactivation (fold), whereas results in Figures 4A and 4B were carried out at the same time. Significant ($p < 0.05$) enhancement of coactivation is indicated by an asterisk.

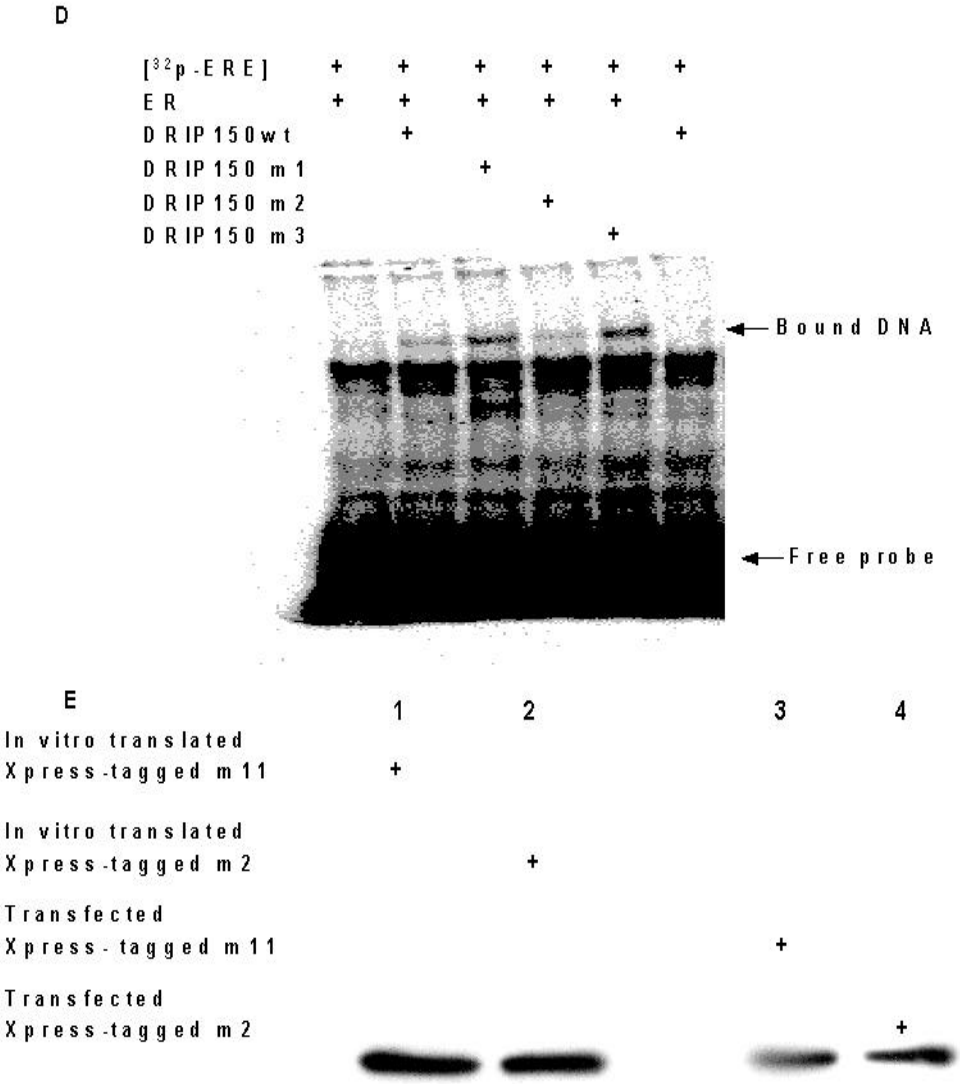

FIG. 31. Continued. (D) Gel mobility shift assay. [^{32}P]ERE and ERα were incubated with equal amounts of *in vitro* expressed wild-type or variant DRIP150 and analyzed in a gel mobility shift assay as described in the Materials and Methods. The specifically-bound retarded band (bound DNA) is indicated. (E) Expression of DRIP150 mutants. DRIP150m11 and DRIP150m2 (expressed tagged) were *in vitro* translated (lanes 1 and 2) or transfected into COS7 cells (lanes 3 and 4), and aliquots of translated protein or whole cell lysates were analyzed by SDS-PAGE and immunoblot analysis as described in the Materials and Methods.

DRIP150 expression plasmid; however, similar results were observed for DRIP150 mutants over a range of plasmid concentrations in separate experiments (data not shown). Coactivation of ERα was further investigated with a series of DRIP150 mutants with deletions of aa 1454-977 (DRIP150m4), 1454-886 (DRIP150m5), 1454-870 (DRIP150m6), and 1454-865 (DRIP150m7). The results summarized in Figure 31B demonstrate that all of the mutants coactivated ERα in ZR-75 cells transfected with $pERE_3$, and these were also observed with different amounts of expression plasmid. A VXXLL motif was present in DRIP150m6 but not DRIP150m7; however, the activity of both mutants as coactivators of ERα suggests that the VXXLL motif was not required for coactivation by the DRIP150 mutant constructs. Results summarized in Figures 31A and 31B demonstrate that the C-terminal NR box of DRIP150 is not required for coactivation and that amino acids 864-789 are necessary for coactivation.

An additional series of DRIP150 mutants containing deletions of aa 1454-850 (DRIP150m9), 1454-827 (DRIP150m10), 1454-812 (DRIP150m11), and 1454-865/77-1 (DRIP150m12) were also investigated as coactivators of ERα (Fig. 31C). The activities of these constructs were determined in separate experiments where there was some variability in the fold induction by E2 and the amount of mutant DRIP150 expression plasmid required to give maximal coactivation. Therefore, data obtained for these constructs are reported as fold enhancement of coactivation compared to cells treated with E2 alone (no coactivation). The results showed that all the DRIP150 deletion constructs coactivated ERα. The deletion of the N-terminal NR box (DRIP150m12) did not result in loss of coactivation, showing that this motif was not necessary for DRIP150 coactivation of ERα. Thus, results of deletion analysis of DRIP150 indicates that the 23 amino acids between aa 811-789 were required for coactivation of ERα in ZR-75 cells.

We also investigated interactions of ERα and [^{32}P]ERE in the presence or absence of *in vitro* expressed DRIP150 expression plasmids (Fig. 31D). The ERα-ERE retarded band (lane 1) intensity was enhanced after coincubation with wild-type DRIP150 (lane 2) and deletion mutants m1, m2 and m3 (lanes 2 - 4). Wild-type DRIP150 alone did not bind [^{32}P]ERE (lane 5), and ERα antibodies (lane 7) but not IgG (lane 6) supershifted the retarded band as indicated in Figure 30B. The enhanced ERα-ERE retarded band intensity was observed after coincubation not only with wild-type DRIP150 and mutant m1 which coactivate ERα, but also with mutants m2 and m3 that are inactive as coactivators. These results suggest that this response may reflect interactions of DRIP150 mutants with ERα *in vitro* but these interactions did not predict their activities as coactivators of ERα. DRIP150m2 and DRIP150m11 have similar molecular weights as illustrated in Figure 31E in which *in vitro* expressed DRIP150 mutants were analyzed by SDS-PAGE and Western blot analysis (lanes 1 and 3). These proteins were also observed in whole cell lysates after transfection (lanes 3 and 4) in COS7 cells.

Coactivation/squelching by DRIP150 coactivation peptide

DRIP150m8 plasmid expresses amino acids 885-755 which encompass the region of DRIP150 required for coactivation of ERα. Results in Figure 32A show that DRIP150m8 coactivates ERα at low concentrations and squelches activity at higher concentrations. Moreover, results summarized in Figure 32B show that DRIP150m8 inhibits coactivation of ERα by wild-type DRIP150. DRIP150 and other deletion mutants also coactivate HE19 and the results in Figure 32C show that DRIP150m11 coactivates ERα and cotransfection with DRIP150m8 inhibits or squelches the coactivation

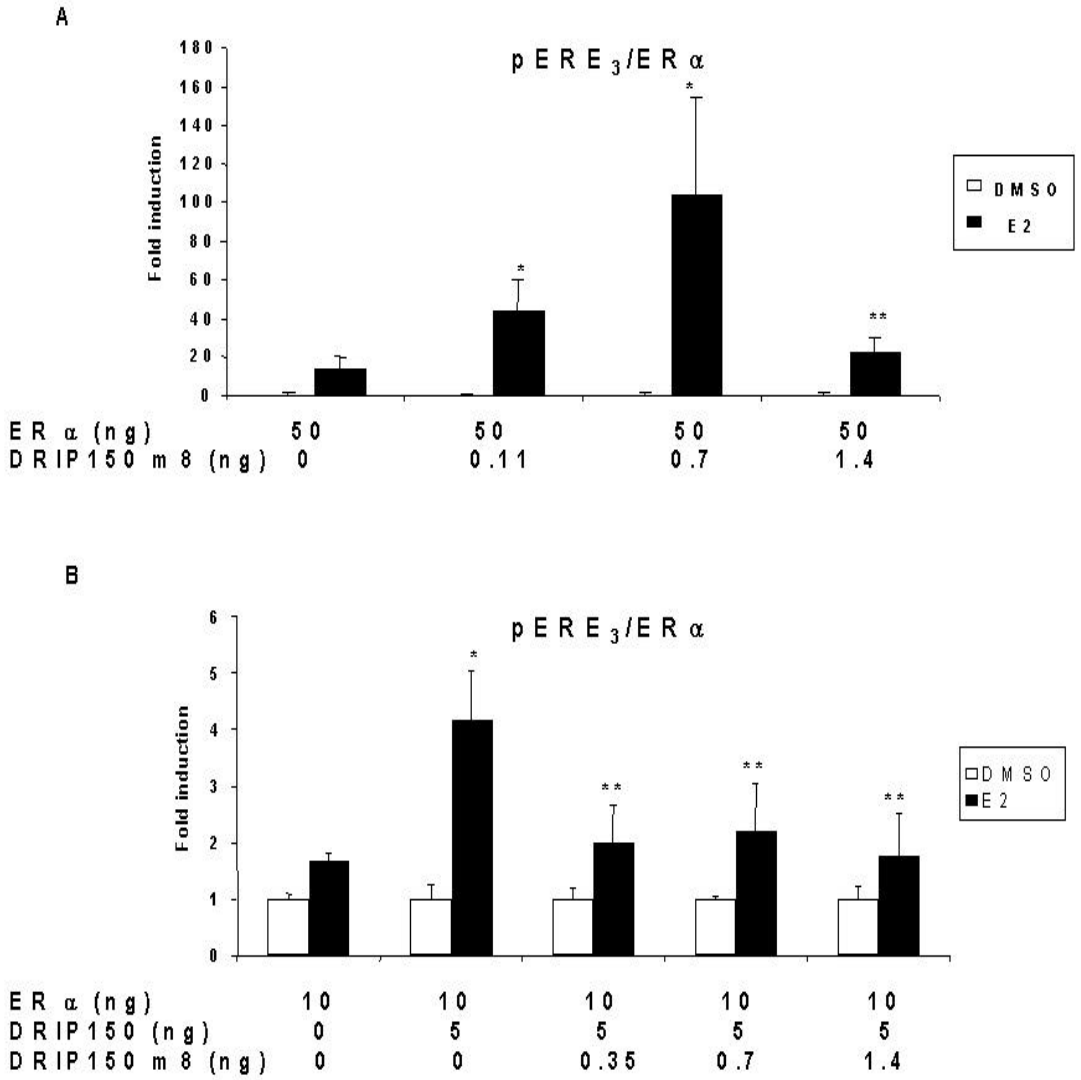

FIG. 32. **Coactivation and squelching by DRIP150m8.** (A) Coactivation of ERα. ZR-75 cells were treated with DMSO or 10 nM E2 transfected with $pERE_3$, ERα (50 ng), and different amounts of DRIP150m8 expression plasmid, and luciferase activity was determined as described in the Materials and Methods. Results of all experiments illustrated in this Figure are means ± SE for at least three experiments for each treatment group, and significant ($p < 0.05$) coactivation (*) and inhibition of coactivation (**) are indicated. (B) DRIP150m8 squelching of ERα. ZR-75 cells were treated with DMSO or 10 nM E2 and transfected with $pERE_3$, ERα (10 ng), DRIP150 (5 ng), and different amounts of DRIP150m8 expression plasmid, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) coactivation by DRIP150 (*) and inhibition by cotransfection with DRIP150m8 (**) are indicated.

C

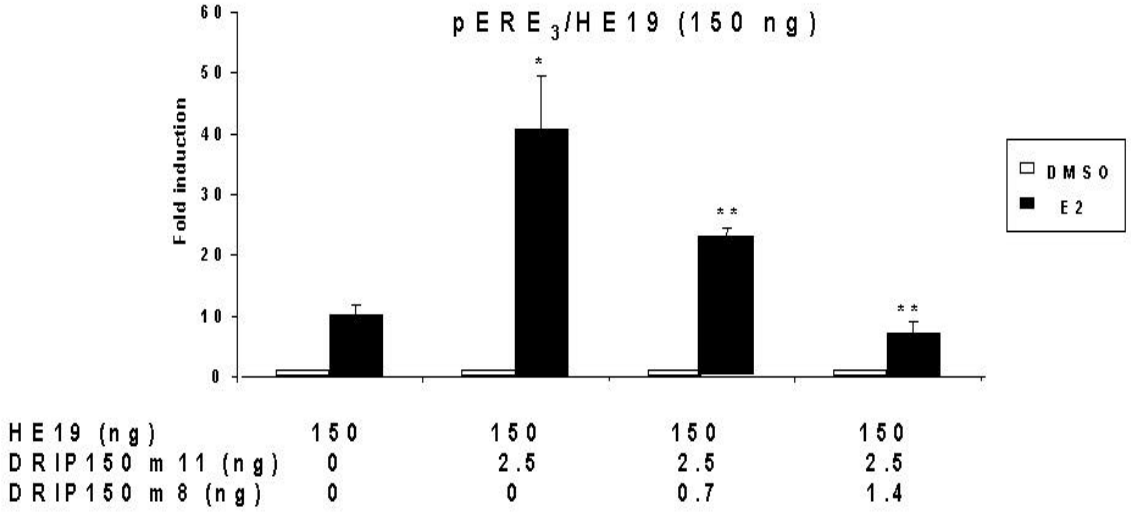

FIG. 32. Continued. (C) DRIP150m8 squelching of HE19. This experiment was carried out as described in Figure 5B, except that HE19 expression plasmid was used.

response. pM23 contains the minimal sequence of DRIP150 (aa 811-789, DIPAHLNIFSEVRVYNYRKLILC) necessary for coactivation of ERα, and this peptide is fused to the DBD of the yeast GAL4 protein (Fig. 33A). Transfection of ZR-75 cells with $pERE_3$ and different amounts of pM23 expression plasmid showed that this chimeric protein coactivates ERα and then squelches this response with increasing amounts of transfected plasmid (Fig. 33A). This parallels a similar coactivation/squelching response observed for DRIP150m8 (Fig. 32A). Moreover, pM23 also inhibits DRIP150 coactivation of ERα (Fig. 33B) demonstrating that pM23 and DRIP150m8 exhibit comparable coactivation of ERα at low concentrations but also squelch transactivation (at higher concentrations) and inhibit DRIP150 coactivation of ERα. We also examined the protein crystal structure database for similarities between the DRIP150 amino acid sequence 811-789 with other proteins. The first six residues DIPAHL fold into an α-helix when they occur in Lanuginosa lipase (419) and histamine N-methyltransferase (420). There was also homology between residues 7 - 16 (NIFSEVRVYN) of the DRIP150 23 amino acid sequence and an α-helical region in hepatocyte nuclear factor 1 (HNF1; NLVTEVRVYN) (421). Results in Figures 33C and 33D summarize squelching experiments with pM23R801P and pM23A792P which express the GAL4-23 amino acid fusion protein with mutations at amino acids 801 (R → P) and 792 (A → P). Proline residues were inserted to disrupt α-helical structure. The results show that pM23R801P did not squelch DRIP150 coactivation of ERα (Fig. 33C), whereas pM23A792P exhibited wild-type (pM23) activity and squelched DRIP150 coactivation of ERα (Fig. 33D). Squelching of DRIP150 coactivation of ERα was also not observed using the double mutant pM23A792P/R801P (Fig. 33E). These data suggest that the sequence at

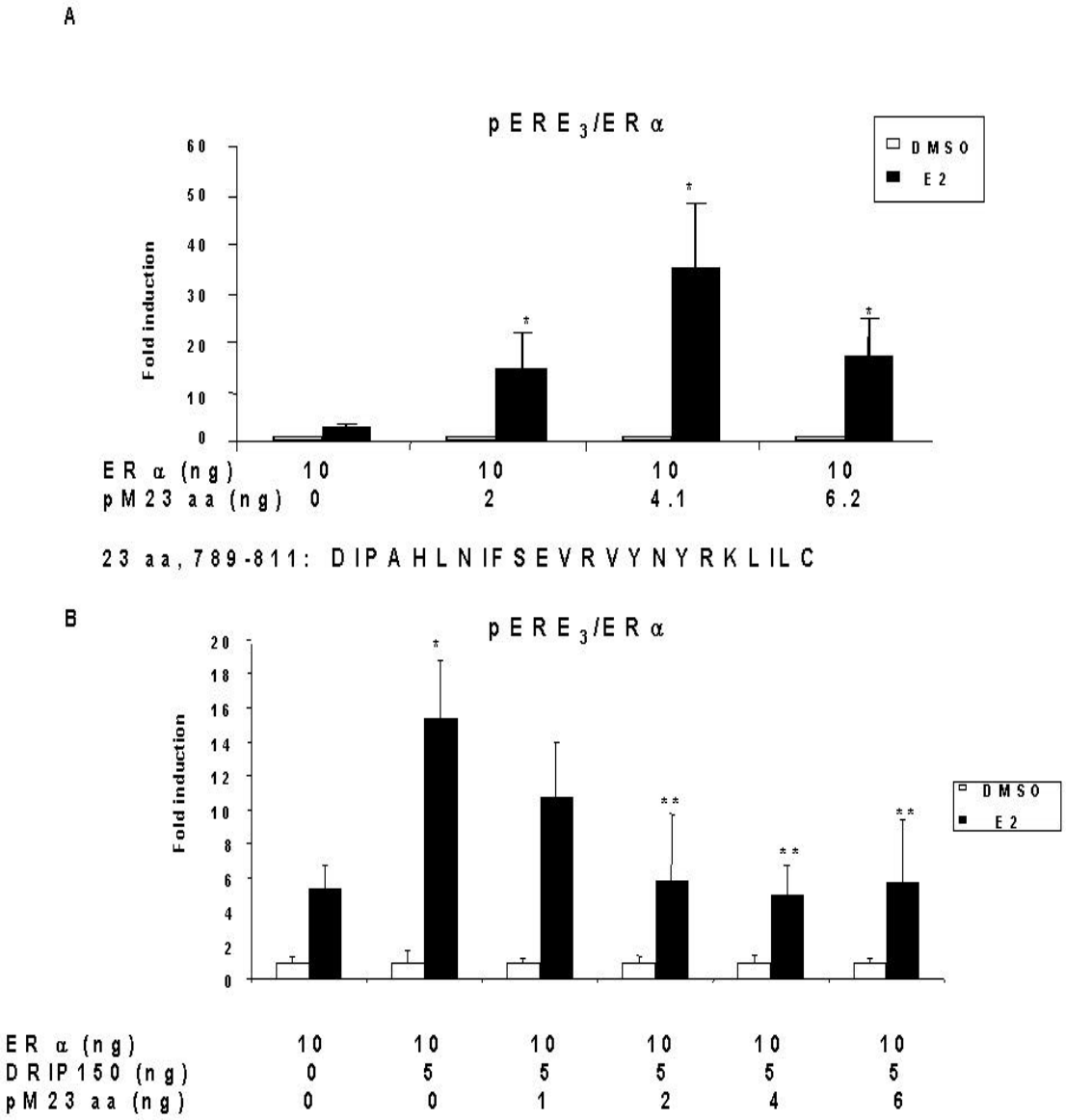

FIG. 33. **Wild-type and variant pM23 as a coactivator and inhibitor of ERα-mediated transactivation.** (A) pM23 coactivates/squelches ERα. ZR-75 cells were treated with DMSO or 10 nM E2, transfected with $pERE_3$, ERα (10 ng), and different amounts of pM23 expression plasmid, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction (*) and squelching (**) are indicated. (B) pM23 squelches coactivation of ERα by DRIP150. ZR-75 cells were treated as described in Figure 33A and DRIP150 (5 ng), ERα (10 ng) and pM23 (1 - 6 ng) expression plasmids were used as indicated. Significant ($p < 0.05$) coactivation by DRIP150 (*) and squelching by pM23 (**) are indicated.

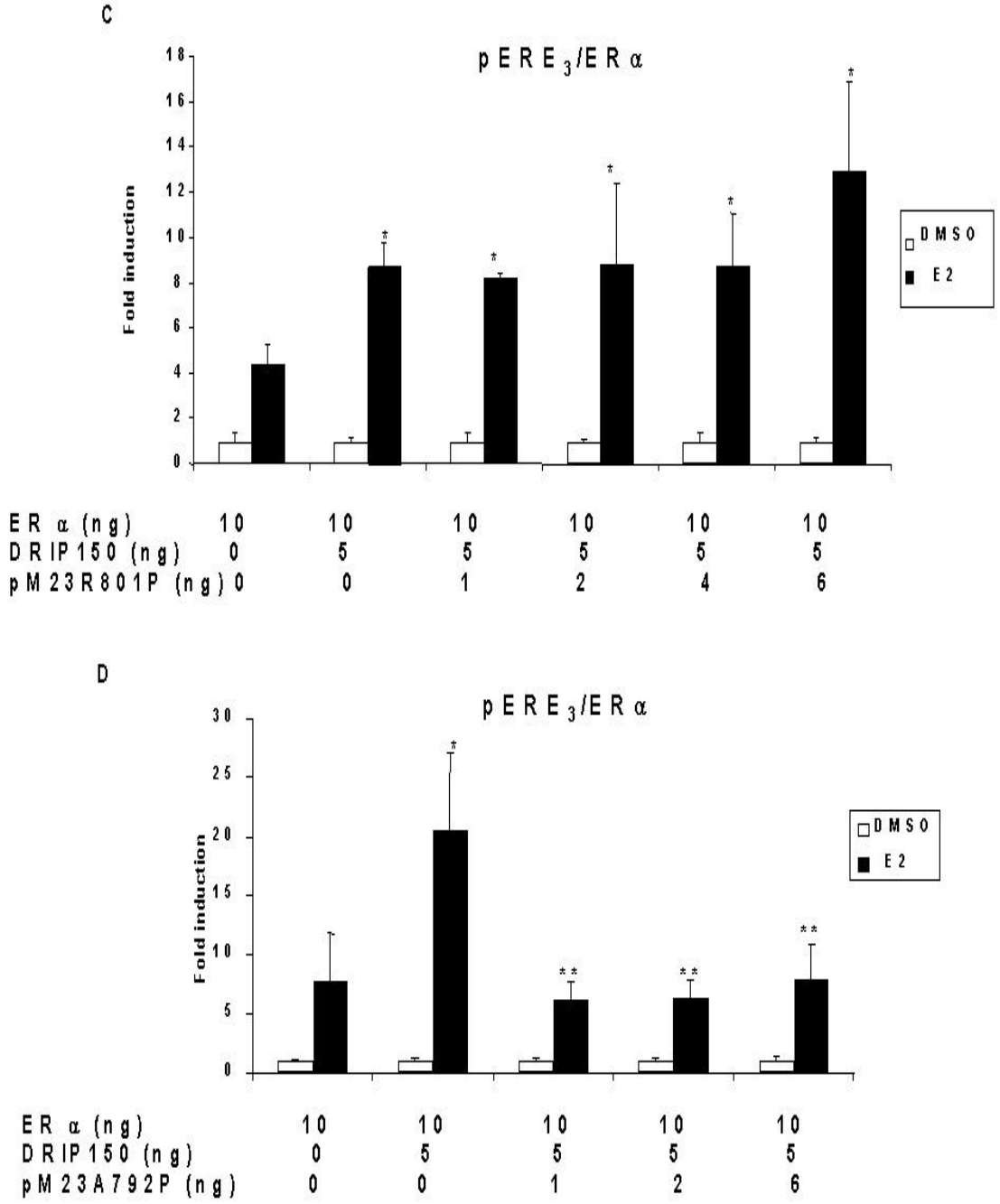

FIG. 33. Continued. Squelching of DRIP150 coactivation by pM23R801P (C), pM23A792P (D) or pM23A792P/R801P (E). ZR-75 cells were treated with DMSO or 10 nM E2, transfected with $pERE_3$, ERα (10 ng), DRIP150, and different amounts of pM23 mutant expression plasmids. Significant ($p < 0.05$) coactivation by DRIP150 (*) and squelching (**) by mutant pM23 constructs are indicated.

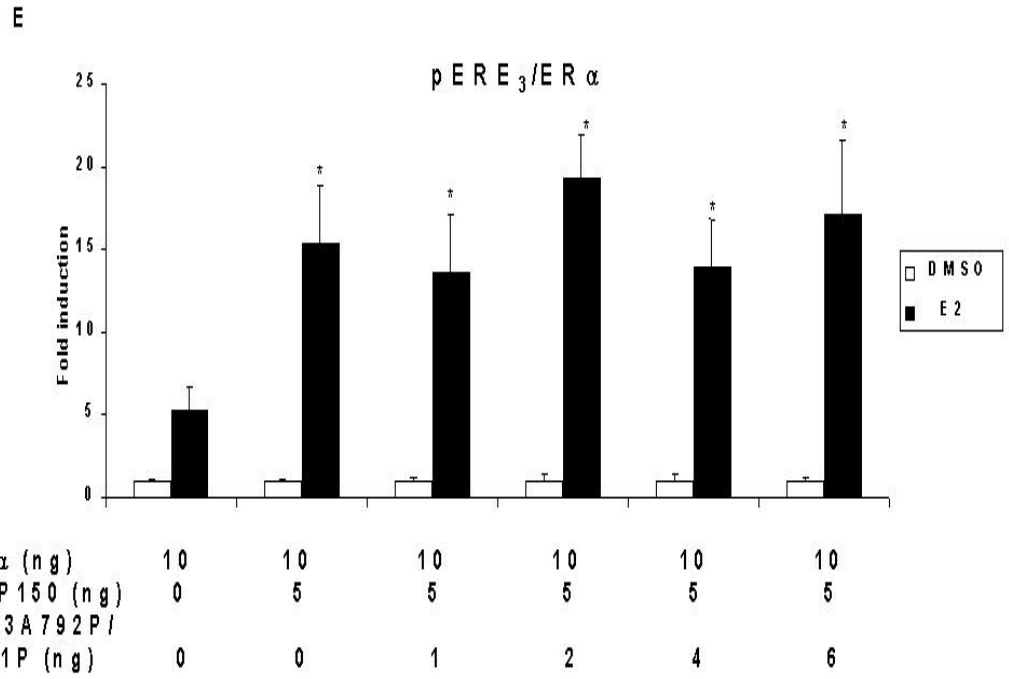

FIG. 33. Continued.

amino acids 795-804 in DRIP150 which resembles an α-helical motif in HNF-1 is an important structural feature of DRIP150 required for coactivation of ERα. These data suggest that in addition to LXXLL motifs, other helical sequences in coactivators can play a role in coactivation of ERα and possible other nuclear receptors in breast cancer cells.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

3',4'-DMF as an AhR antagonist in human breast cancer cells

The AhR is a ligand-activated transcription factor that binds structurally diverse chemicals that include highly toxic halogenated aromatics (411) and chemoprotective phytochemicals such as I3C, DIM and bioflavonoids (132,146,234). Research in this laboratory identified a series of alternate-substituted (1,3,6,8- or 2,4,6,8-) alkyl PCDFs typified by the AhR antagonist 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) that inhibited TCDD-induced CYP1A1, porphyria, immunotoxicity and cleft palate formation in mice (223,422,423). Subsequent studies showed that MCDF was an AhR agonist and inhibited E2-induced responses in the rodent uterus and breast cancer cells and mammary tumor growth in carcinogen-induced female Sprague-Dawley rats (221,222,227,424,425). It has also been reported that synthetic and natural flavonoids exhibit AhR antagonist activity and some of the most effective AhR antagonists are substituted 3'-methoxyflavones (173,180,181,214,215).

3'-Methoxy-4'-nitro- and 4'-amino-3'-methoxyflavone have been extensively characterized as AhR antagonists that act by inhibiting formation of the nuclear AhR complex (173,180,181,214), and similar results have been observed for 2'-amino-3'-methoxyflavone (215,216). However, many of these substituted flavones are also protein tyrosine kinase inhibitors and cytotoxic at doses >10 μM (193,215,216). Henry and coworkers (1999) recently investigated a series of 3'-methoxy-4'-substituted flavones and showed that the most active AhR antagonists contained 4'-substituents with high electron density (nitro, azido and thiocyanate). It was hypothesized that this

structural feature facilitated critical hydrogen-bonding with the AhR. In contrast, 3'-methoxy-4'-aminoflavone was less active as an AhR antagonist (181) than previously observed in this laboratory in breast cancer cell lines (173), and this may be due, in part, to cell context. We also observed that although 4'-methoxyflavone did not inhibit induction of CYP1A1 by TCDD, this compound blocked TCDD-induced transformation of the rat cytosolic AhR (214), and we therefore hypothesized that 3',4'-DMF, which contains two vicinal methoxy groups, may be an effective AhR antagonist in breast cancer cell lines despite the lack of a 4'-substituent with high electron density.

Most previous studies have characterized the activity of AhR antagonists by determining their effects on CYP1A1-dependent (gene/promoter) activities and their interactions with the cytosolic and nuclear AhR complex. Results obtained for 3',4'-DMF in breast cancer cells are similar to those previously reported for other 3'-methoxyflavones in different cell lines indicating that inhibition of TCDD-induced CYP1A1 or related activities is due to competitive interaction of the ligand with the cytosolic AhR that does not undergo transformation or nuclear translocation (Fig. 20). 3',4'-DMF did not block inhibition of E2-induced proliferation by TCDD (data not shown) and this may be related to the growth inhibitory effects of 3',4'-DMF alone (Fig. 18) which could be due to partial ER antagonist activities or inhibition of protein kinase dependent pathways (193,215). However, in transcriptional activation assays using the E2-responsive pCKB construct, 3',4'-DMF exhibited AhR antagonist activity and reversed the antiestrogenic effects of TCDD in this assay (Fig. 19). Thus, 3',4'-DMF does not resemble MCDF which acts as an AhR agonist for this response (409) but resembles α-naphthoflavone which inhibits AhR-mediated CYP1A1 induction (210,212) and inhibition of E2-induced transactivation in breast cancer cells (208). The major

advantage of 3',4'-DMF over α-naphthoflavone is that the latter compound is an AhR antagonist at concentrations < 1 μM, whereas at higher concentrations (1 - 10 μM), α-naphthoflavone is an AhR agonist (213). Results obtained in this study demonstrate that 3',4'-DMF inhibits both AhR-mediated CYP1A1 induction and antiestrogenic activity in breast cancer cell lines by blocking transformation of the cytosolic AhR complex and formation of the nuclear AhR complex, and this is consistent with results of previous studies with other 3'-methoxy-substituted flavones (173,180,181,214-216). Previous studies demonstrated that 3'-methoxyflavone exhibited minimal activity as an inhibitor of TCDD-induced transformation of rat hepatic cytosolic receptor, whereas introduction of high electron density 4'-substituents gave compounds that inhibited TCDD-induced transformation and transcription in mouse liver cells (181). In contrast, 3'-methoxyflavones that contain 4'-methoxy (this study) and 4'-amino groups (173) that do not have high electron densities are also relatively potent AhR antagonists in breast cancer cells suggesting that the AhR antagonist activity of these substituted flavones may be influenced by cell context. An important advantage in using 3',4'-DMF is the commercial availability of this compound.

Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells

Resveratrol exhibits a diverse spectrum of biochemical responses that may be linked to health benefits associated with moderate consumption of wine (426-428). Several recent studies have demonstrated that resveratrol decreases CYP1A1 mRNA/protein or related activities in cell culture and *in vivo* and there is some evidence that this inhibitory response may be related to AhR antagonist properties of this compound (266-269). At least two major classes of AhR antagonists have been

identified, and these include 3'-methoxy-substituted flavones that block receptor transformation and formation of the nuclear AhR complex (173,180,181) and alternate substituted polychlorinated dibenzofurans (PCDFs). These latter compounds, typified by 6-methyl-1,3,8-trichlorodibenzofuran, compete with TCDD for binding the AhR but form a nuclear AhR complex that only weakly activates DRE-dependent responses such as induction of CYP1A1 gene expression (222,409). Resveratrol also inhibits CYP1A1 gene expression and related enzyme activities, and it has been suggested that resveratrol is also an AhR antagonist (266,267,269).

Our results also show that resveratrol inhibits TCDD-induced CYP1A1 mRNA and related enzyme activity in breast cancer cells; however, interactions of resveratrol with the AhR suggest that these responses may be non-specific. Treatment of T47D or MCF-7 cells with resveratrol resulted in formation of a nuclear AhR complex as determined in gel mobility shift assays, and interactions with TCDD did not markedly affect nuclear AhR complex formation (Fig. 24). These results were consistent with a previous report using breast cancer cells (267) but were in contrast to studies in HepG2 cells (266,269) which showed that treatment with resveratrol alone did not result in formation of a nuclear AhR complex and, in cotreatment studies (resveratrol + TCDD), resveratrol inhibited TCDD-induced formation of the AhR complex. Differences observed in this assay may be due to cell type (breast vs. liver) and, therefore, interactions of resveratrol were further investigated using rat hepatic cytosol which is readily transformed by TCDD. Induced transformation is inhibited by pure AhR antagonists such as 3'-methoxy-4'-nitroflavone (Fig. 24C) (173,180,181), whereas resveratrol-induced transformation was concentration-independent and resveratrol plus TCDD gave minimal interactions in the assay (Fig. 24). Seidel and coworkers (2000)

have also reported that like resveratrol, several structurally different compounds induced transformation of guinea pig hepatic cytosolic AhR but did not exhibit functional AhR agonist activity. These data, coupled with the failure of resveratrol to exhibit AhR agonist activity in functional assays and AhR antagonist activity in cells transfected with Ah-responsive pRNH11c (Fig. 25), suggest that resveratrol-AhR interactions may be non-specific.

Nevertheless, results of this study and previous reports (266-269) clearly show that resveratrol decreases CYP1A1 mRNA and protein levels, and the latter response is not related to proteosome-dependent degradation of CYP1A1 protein (Fig. 27). Therefore, we further investigated interactions between resveratrol and downregulation of CYP1A1 by determining post-transcriptional effects on CYP1A1 mRNA stability. A recent report showed a remarkable similarity between resveratrol and DHEA which both inhibited induction of CYP1A1 mRNA and EROD activity in breast cancer cells (429), and we also observed these responses for resveratrol (Fig. 22) and DHEA (data not shown). Moreover, like resveratrol (Fig. 25), DHEA did not inhibit induction in cells transfected with an Ah-responsive element (429). Our results (Fig. 26) also showed that both DHEA and resveratrol increased the rate of CYP1A1 mRNA degradation in T47D cells suggesting that resveratrol (like DHEA) also inhibits CYP1A1 expression by post-transcriptional mechanisms. The importance and specificity of AhR-independent actions of resveratrol are unknown; however, a recent study reported that resveratrol also decreased androgen receptor mRNA levels in LNCaP prostate cancer cells (430). It is possible that these effects may also be linked to a post-transcriptional AhR-independent mechanism as reported in this study, and the role of this pathway in mediating

resveratrol action, particularly in hormone-responsive cancer cells, is currently being investigated.

DRIP150 coactivation of ERα in ZR-75 breast cancer cells is independent of LXXLL motifs

Several nuclear coregulatory complexes that associate with transcription factors and potentiate RNA pol II transcription have been identified and many of their individual subunits are identical (391,392,396,398,399,432,433). The functions of the DRIP, TRAP, NAT, ARC and CRISP coregulatory complexes are similar to that described for Mediator complexes initially purified from yeast. Interactions of these coregulatory complexes with NRs including ERα and ERβ have been investigated, and there is evidence in some cell lines that DRIP205 anchors the protein complex to NRs (391,392,415,434). Several reports have investigated DRIP205 coactivation of NRs including both ERα and ERβ. DRIP205-dependent coactivation of ER depends on both cell context and ER-subtype and the NR boxes of DRIP205 are required for coactivation (435-440). Both DRIP205 and DRIP150 also directly interact with ERα/ERβ, and other studies confirm that DRIP150 interacts with the glucocorticoid and androgen receptors (393,394,415).

Previous studies indicate that DRIP150 coactivated glucorticoid receptor (GR)-mediated transactivation and this response was AF1-dependent (394); however, coactivation of ERα by DRIP150 has not been extensively investigated. DRIP150 coactivates ERα in MDA-MB-231 and ZR-75 cells transfected with $pERE_3$ and comparable enhancement of transactivation was observed in a mammalian one-hybrid assay in cells transfected with pM-ERα/GAL4-luc (Fig. 28). DRIP150 also coactivated HE19 but not ERα-TAF1 in ZR-75 cells, suggesting that the AF1 domain of ERα was

not necessary for coactivation and that an intact helix 12 was required. These results contrast to the reported AF1-dependent coactivation of GR by DRIP150 (393) but are comparable to previous studies in several different cell lines showing that helix 12 is a critical surface of ERα which interacts with NR boxes of p160 coactivators (382,385,441-443). The importance of the AF2 region of ERα for coactivation by DRIP150 was supported by the inhibitory effects or squelching of enhanced transactivation in ZR-75 cells transfected with an NR box expression plasmid (Fig 29A). This construct contains two copies of the GRIP1 NR box and inhibits ERα-mediated transactivation (387). In contrast, overexpression of an AF1 peptide (aa 1-182 of ERα) did not affect coactivation of ERα by DRIP150 confirming the important role of AF2 of ERα.

DRIP150 contains 2 LXXLL NR box motifs in the N- (73-69) and C-terminal (1186-1182) regions. Their role in coactivation of ERα by DRIP150 has not been determined; however, previous studies show that NR boxes are critical regions for the coactivation of NRs by DRIP205 (436,437,444). In contrast, deletion analysis of DRIP150 (Figs. 31 and 32) clearly shows that coactivation of ERα by DRIP150 deletion variants was NR box-independent, and a 23 amino acid sequence (aa 811-789) was identified as an essential region for DRIP150 coactivation of ERα (Fig. 31). Wild-type DRIP150 coimmunoprecipitates ERα (Fig. 30A) as previously reported (415); however, in gel mobility shift assays DRIP150 does not form a DRIP150/ERα/ERE ternary complex but enhances the ERα-ERE retarded band intensity. The failure to observe a supershifted ternary complex is not unprecedented since previous studies report that ERα enhances Sp1/Sp3 DNA binding (100,445), cyclin D1 enhances ERα DNA binding,

human T-cell lymphotropic virus Type I transcriptional activator (Tax) enhances CREB DNA binding and binding of other transcription factors in gel mobility shift assays (416-418). Interestingly, the results also show that DRIP150 and DRIP150m1 which coactivate ERα also enhance the ERα-ERE retarded band; however, mutants that are inactive as coactivators (DRIP150m2 and DRIP150m3) exhibit comparable activity in the gel shift assay (Fig. 31D). This suggests that enhancement of ERα-ERE binding by DRIP 150 variants is not predictive for coactivation of ERα-mediated transactivation which requires the 23 amino acid 811-789 sequence.

We have further investigated the role of the DRIP150 "coactivation sequence" in hormone-induced transactivation using DRIP150m8 which contains amino acids 885-755 and pM23 which contains DRIP150 amino acids 811-789 fused to the yeast GAL4-DBD. Transfection of either protein gave a biphasic response typical of many coactivators in which low concentrations resulted in coactivation of ERα and higher amounts of transfected plasmids subsequently decreased or squelched transactivation (Fig. 32). pM23 and/or DRIP150m8 also inhibit wild-type and mutant DRIP150 coactivation of ERα or HE19 (Fig. 32) and these results resemble responses observed for NR box peptides containing LXXLL sequences (387). Our results confirm that DRIP150 interacts with ERα as previously reported (415) and coactivates ERα in ZR-75 (and MDA-MB-231) cells transfected with $pERE_3$. The coactivator activity of DRIP150 alone in ZR-75 cells contrasts to previous reports showing that ligand-dependent recruitment of mediator complex proteins to ERα and other nuclear receptors requires DRIP205 as an anchor component for complex-receptor interactions (391,392,415,434). However, other reports show that DRIP150 alone interacts with nuclear receptors (393,394) and this has been observed for ERα (Fig. 30A). A recent study also reported

isolation of a transcriptionally active coactivator CRSP/mediator complex that contained CRSP150/DRIP150 but not DRIP205/Med220 (or Med70), suggesting that DRIP205 is not always required for a functional mediator coactivator complex (446). This is also supported, in part, by chromatin immunoprecipitation studies on the time-dependent recruitment of coactivators, such as SRCs and DRIPs, to the ERE of the pS2 gene promoter in MCF-7 cells (435). The results showed that at some time points, DRIP150 was associated with the pS2 promoter in the absence of DRIP205, suggesting a DRIP205-independent role for DRIP150 as a coactivator of ERα and this is consistent with results of this study.

DRIP150 coactivation of ERα is independent of the two NR boxes and requires a 23 amino acid sequence DIPAHLNIFSEVRVYNYRKLILC at 789-811 (Figs. 31 and 32). Using the protein crystal structure database, there was not a good match between the 23 amino acid DRIP150 sequence and other known crystalline proteins; however, the first six residues DIPAHL fold into an α-helix when they occur in Lanuginosa lipase and histamine N-methyltransferase (419,420). Amino acids 7-16 in DRIP150 are homologous to amino acids 69-78 in hepatocyte nuclear factor 1 which also fold into an α-helix (421). pM23 efficiently squelches DRIP150 coactivation of ERα (Fig. 33B), and we used this assay to identify the function of the two helical components within the 789-811 amino acid region of DRIP150. Results in Figure 33 show that pM23A792P exhibited wild-type (pM23) squelching activity, whereas, pM23R801P and pM23A792P/R801P (double mutant) did not squelch coactivation of ERα by DRIP150. These data suggest that the α-helical structure within the NIFSEVRVYN (amino acids 795-804) sequence is required for the activity of DRIP150 as a coactivator of ERα. Results of this study uniquely identify a novel sequence in DRIP150 required for

coactivation of ERα and demonstrate that LXXLL boxes in DRIP150 are not required for enhancement of ERα-dependent transactivation. Current studies are focused on the function of DRIP150 and the 789-811 amino acid sequence in coactivation of ERα/Sp1- and ERα/AP1-mediated transactivation and coactivation of other nuclear receptors.

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