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Molecular Mechanisms Underlying the Action of Environmental Endocrine-Disrupting Chemicals

Hajime Nawata[3]*, Kiminobu Goto, Hidetaka Morinaga[3], Toshihiko Yanase, Jun Yanagisawa[1,3], Shigeaki Kato[1], Masatoshi Nomura, Taijiro Okabe and Ryoichi Takayanagi[2]

Department of Medicine and Bioregulatory Science (3rd Department of Internal Medicine), Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan
[1]Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
[2]Department of Geriatric Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan
[3]CREST, JST

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1. Preface

The reproductive hazards of environmental endocrine-disrupting chemicals (EEDs) to fish, wildlife and humans have been causing alarm among the general public. Increased incidence of hypospadia and retained testis, and decrease in sperm counts in humans have been suspected to be caused by EEDs. The mechanisms of ontogeny and differentiation from stem cells to adult adrenal and gonadal organs (testis and ovary) to produce steroid hormones through the cascade of transcription factors, such as Ad4BP (SF-1), WT-1, DAX-1, SOX-9, androgen receptor (AR) and estrogen receptor (ER), have been recently established (Fig. 1).

Gene mutations of the above mentioned transcription factors induce various sex differentiation abnormalities in humans, which are the same as those seen in wildlife. Therefore, we hypothesized that EEDs interact with these transcription factors and cause sex differentiation abnormalities. We have focused on AR, ER, and steroidogenic cytochrome P450 to clarify the molecular mechanisms of the action of EEDs to disrupt the reproductive process. AR or ER is known to be ligand-specifically activated. For example, after dihydrotestosterone (DHT) binding, AR translocates into the nucleus and then binds

*E-mail: nawata@intmed3.med.kyushu-u.ac.jp

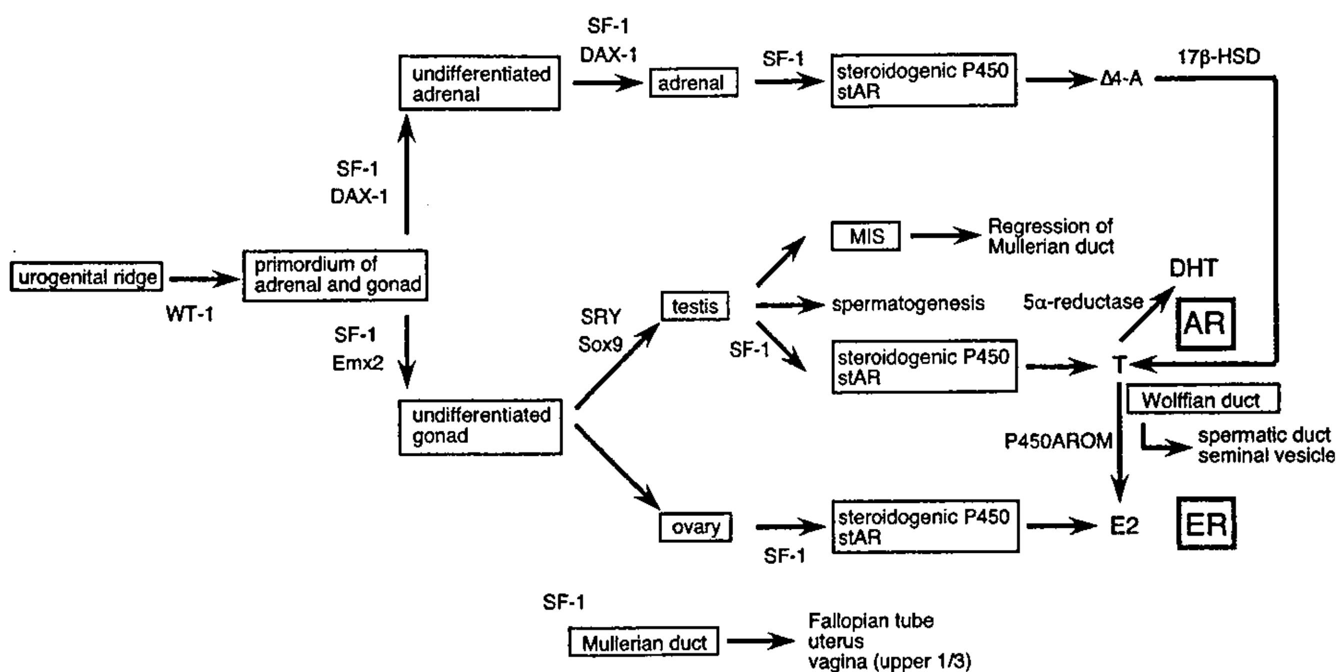

Fig. 1. A cascade of transcription factors involved in development of adrenal and genital organs.

to specific DNA sequences. Subsequently DHT-bound AR activates the transcription of its target genes. AR and ER play an essential role during the differentiation of gonadal tissues, for example, mutations in the AR gene may cause infertility in men. Steroid hormone receptors contain three functional domains: a variable N-terminal transactivating domain (NTD), a highly conserved DNA-binding domain (DBD), and a moderately well-conserved C-terminal ligand-binding domain (LBD). The AR or ER, similar to other members of the steroid hormone receptor superfamily, harbors two transcription activation functions (AFs): a constitutively active AF-1 located in the NTD of the receptor, and a ligand-dependent AF-2 within the LBD. A number of transcription cofactors (coactivator or corepressor) that associate, in a ligand-dependent fashion, with the AF-2 regions of steroid hormone receptors have been identified. They include members of the p160 family, CBP/p300, PCAF/GCN5, TRAPs/DRIPs, PIAS1, HBO, which are organized in multiprotein complexes and facilitate the access of nuclear receptors and the RNA polymerase II core machinery to their target DNA sequences by chromatin remodeling and histone modification.

Many compounds that either mimic or block the transactivation functions of steroid hormone receptors have been produced. When these compounds unexpectedly alter the endocrine system, they are called environmental endocrine-disrupting chemicals (EEDs). EEDs have emerged as a major public health issue because of their potentially disruptive effects on physiological hormonal actions, particularly, in most cases, through the direct interaction with receptors of estrogen or androgen. That is EED mimics or blocks the function of AR or ER. However, since the target molecule(s) of EED may not be restricted to AR or ER itself, the transcriptional cofactors and the subnuclear compartment of these

functional complexes will be the subject of research for a comprehensive understanding of the action of EED (Fig. 2). Furthermore, some EEDs may induce endocrine-disrupting effects through the activation or suppression of the key enzymes involved in either the steroidogenesis in gonadal tissues or the hepatic cytochrome P450 system required for the metabolism of exogenous chemicals.

2. AF-1 Function is Essential for the Transactivation Function of AR

AR has been thought to be quite unique among members of nuclear receptor superfamily, since most, if not all, of its activities are mediated via the constitutive activity of the AF-1 function. This concept is supported by results of the molecular analysis of a patient in which lack of binding of an as yet unknown but indispensable specific transcription coactivator to the AF-1 fragment of AR resulted in the complete form of the androgen insensitivity syndrome.[1] Direct sequencing of the PCR products of the exons of the AR gene and the coding region of the AR cDNA prepared from the patient's fibroblasts revealed no mutations in the androgen receptor gene. Detailed analyses of androgen action in the patient's fibroblasts indicated that the transactivation signal from AF-2 of the androgen receptor was transmitted normally to the basal transcription machinery, but transmission of the activation signal from AF-1 was disrupted (Fig 3). The glutathione S-

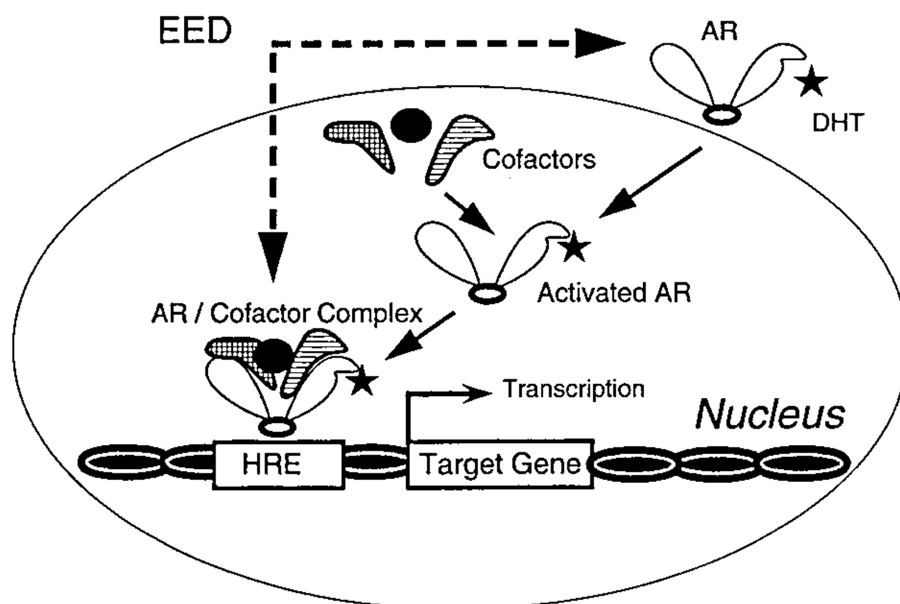

Fig. 2. Action of EED on transcriptional regulation mediated by steroid hormone receptors. In this figure, transactivation of the target gene by AR that translocated into the nucleus after ligand binding is shown. In the nucleus, activated AR forms a huge complex with transcriptional cofactors, then binds to the hormone response element (HRE) to activate the transcription of the androgen target gene. EED may disrupt this transactivation mechanism by either competitive binding to the unliganded AR or, as discussed in the text, altering the higher structure of the AR/cofactor complex possibly by direct interaction with the complex.

transferase (GST) pull-down analysis revealed that proteins with an apparent molecular mass (M_r) of 90 kDa were "pulled down" using the AR-AF-1 from the cellular extracts of the normal fibroblasts, whereas the 90 kDa-protein was not "pulled down" from the extracts of the patient's fibroblasts.

These findings indicated that the AF-1 region of AR may play a fundamental role in the transactivation function of the receptor, and that the identification of a protein(s) bound to AF-1 is essential for a better understanding of the mechanisms of the signal transduction mediated by AR. Thus we employed a yeast two-hybrid screening using the human AR AF-1 sequence as a bait protein, and isolated one sequence named ANT-1 (AR NTD binding protein-1). Surprisingly, the sequence was identical to that of a protein bound to one splicing factor, snRNP (small nuclear ribonucleoprotein particles), suggesting that the transcription/splicing coupling on the AR target gene is evoked in a receptor-specific manner (Fig. 4, paper in preparation).

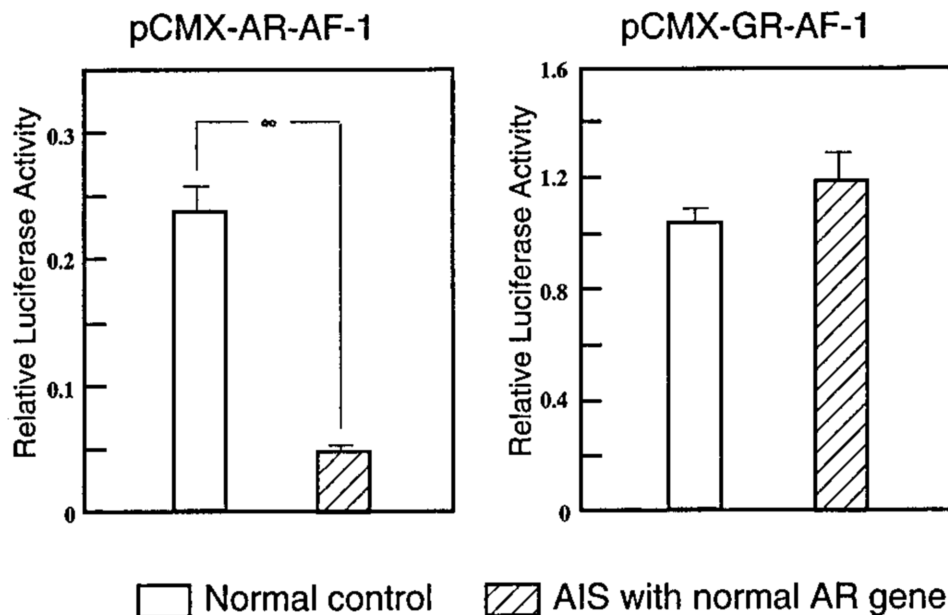

Fig. 3. AF-1 transactivation function in the genital skin fibroblast from a patient who was diagnosed phenotypically and endocrinologically as being complete androgen insensitivity syndrome (AIS), in spite of the presence of normal AR gene. A 19-year-old female complained of primary amenorrhea. Endocrinological examination performed before resection of the testes revealed that both the basal and the chorionic gonadotropin-stimulated levels of serum testosterone and 5α-DHT were within the normal range of adult males. The patient had a normal 46, XY karyotype and was diagnosed as having complete AIS. A luciferase reporter plasmid was transfected with a plasmid that expresses AR or GR lacking LBD (AF-2 domain) into the primary cultured genital skin fibroblast from either a patient (hatched bar) or a normal control subject (open bar). In the patient's fibroblast, constitutive AF-1 function was impaired specifically in AR but not in GR.

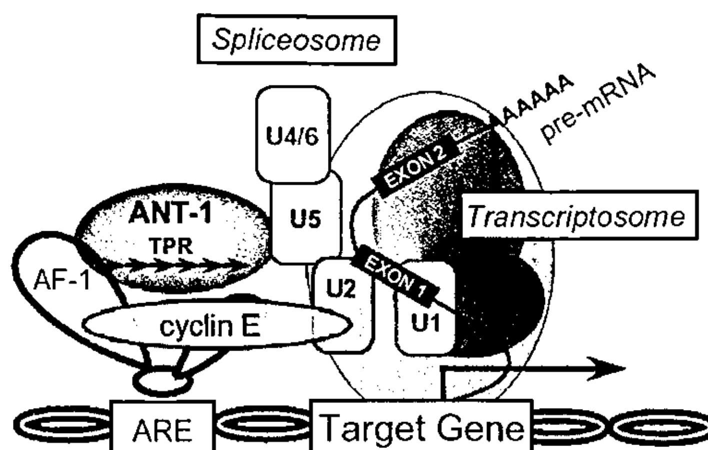

Fig. 4. Interaction of AR-AF1 domain with a transcriptional coactivator, ANT-1. ANT-1 is a U5 snRNP-binding protein, while cyclin E bridges U2 snRNP and AR-AF1 domain.

3. High-Resolution Three-Dimensional Confocal Microscopy Image Analysisis Effective for Screening Antiandrogenic EEDs

Nonsteroidal hydroxyflutamide (OHF) and bicalutamide (CAS) specifically suppress the androgen-dependent transactivation function of AR by competitive binding to the hormone-binding domain of AR. They are called pure antiandrogens and are currently used widely for the treatment of patients with advanced prostatic cancers. Chemicals synthesized for use in herbicides or insecticides, such as vinclozolin or *p, p'*-DDE (1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene), are known to act as antiandrogenic EEDs, and thus have been suspected to be a cause of infertility in wildlife.

Much evidence has been accumulated suggesting that the chromatin structure is dynamic and tightly linked to the transcriptional activity. Nuclear staining with DNA dyes, such as DAPI or Hoechst 33342, has been used to discriminate the heterochromatin region from the euchromatin region. To sensitively clarify the difference in the intranuclear distribution between agonist-bound AR and antagonist-bound AR, we developed a novel approach by performing a three-dimensional (3D) reconstruction of the confocal microscopic images of green fluorescence protein (GFP) fluorescence in the nucleus. This method was applied to the screening of antiandrogenic EEDs.(2) COS-7 cells that were transiently transfected with AR fused to GFP were treated with DHT or OHF, and then their nuclei were simultaneously stained with Hoechst 33342. For each confocal image, which was obtained using the Leica TCS-SP system, low-brightness noise rejection and median filter processing were carried out in the blue (chromatin) and green (GFP) channels, respectively. Then the chromatin and the GFP images were extracted and reconstructed in 3D. For the chromatin images that were stained with Hoechst 33342, less dense areas (namely euchromatin regions) were cut off and thus are shown as blank images. Using our procedures to reconstruct the 3D images of the nucleus, the GFP images could be observed at high resolution, thus allowing us to observe all spatial interrelations between chromatin structures.

The above-mentioned method clearly distinguished the subnuclear localization of transcriptionally active AR tagged with green fluorescent protein (AR-GFP) from transcriptionally inactive AR-GFP (Fig. 5). Transcriptionally active AR-GFP mainly produced 250 to 400 fluorescence foci in the boundary region between euchromatin and heterochromatin, while the exact nature of the fluorescent foci in the nucleus remains to be elucidated. Although AR-GFP bound to such antiandrogens as OHF or CAS translocated to the nucleus, they homogeneously spread throughout the nucleus without producing any fluorescence foci. Antiandrogenic EEDs, such as *p, p'*-DDE, vinclozolin, and nitrofen, also disrupted the intranuclear fluorescence foci (Fig. 6). Furthermore, when the cells were treated with both 10^{-9} M DHT and 10^{-6} M vinclozolin, *p, p'*-DDE or nitrofen, the intranuclear GFP cluster formation was strongly disrupted, and was also observed against the homogeneous GFP fluorescence background, thus demonstrating diffusely distributed AR-GFP. For the first time, nitrofen was found to exhibit antiandrogenic activities using this sensitive imaging method. It was originally synthesized as a herbicide, however, it is no longer available commercially because of its suspected carcinogenecity. It is also suspected that nitrofen contamination during in pregnancy may cause congenital diaphrag-

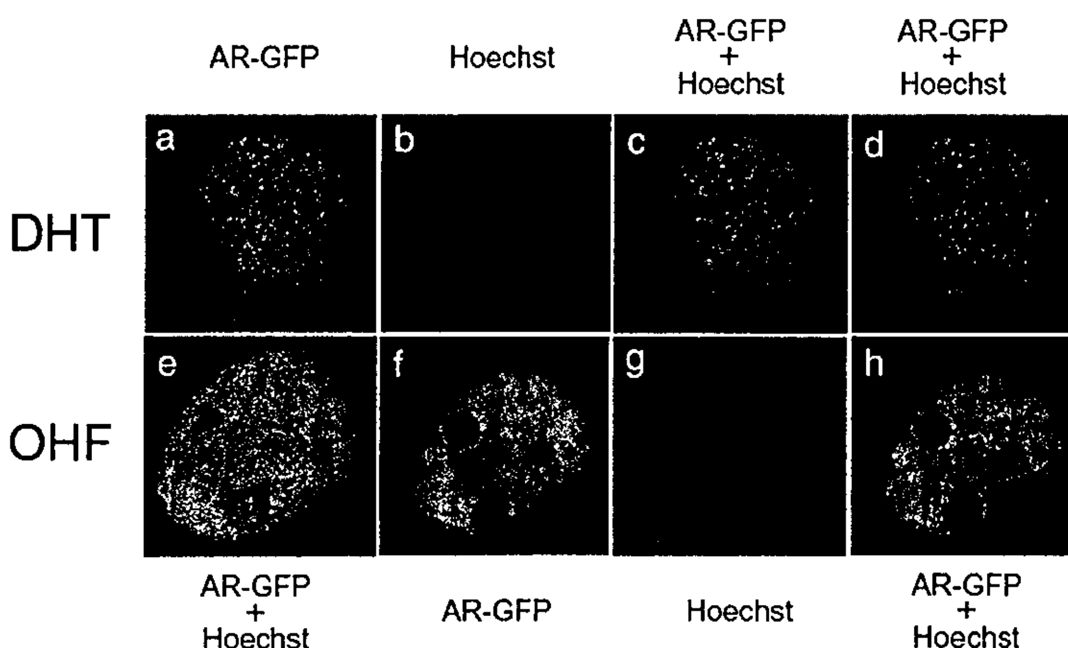

Fig. 5. Three-dimensional image analysis of the intranuclear localization of the agonist- or antagonist-bound AR-GFP. COS-7 cells transfected with a plasmid that expresses the AR-GFP chimeric protein were treated with 10^{-8} M DHT (a-d) or 10^{-6} M OHF (e-h), and were stained with Hoechst33342, and then the confocal images of the nucleus were collected to reconstruct the three-dimensional images. The images were displayed as a surface view (a-c and e), or a tomographic sectional view (d and f-h). a) AR-GFP cluster formation in the nucleus of DHT-treated cells; b) the chromatin structure stained with Hoechst33342; c) spatially superimposed three-dimensional images of a) and b); d) tomographic sectional image of c); e) surface view of the diffuse homogeneous distribution of AR-GFP in the nucleus of OHF-treated cells; f) tomographic view of e); g) chromatin structure stained with Hoechst33342; h) a superimposed image of f) and g).

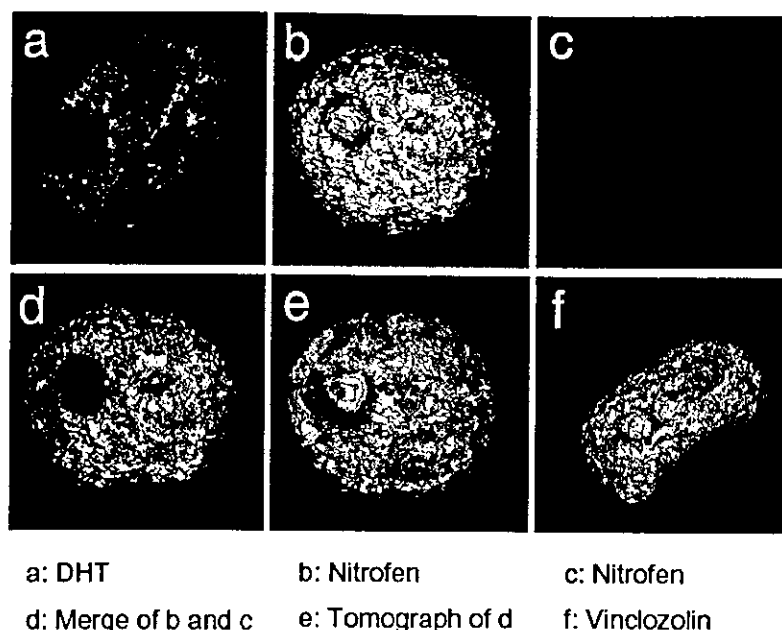

Fig. 6. Images of the intranuclear localization of AR in the COS-7 cells treated with antiandrogenic chemicals. COS-7 cells were transfected with a plasmid that expresses the AR-GFP chimeric protein, and treated with DHT or antiandrogenic EED. Image reconstruction was performed as described in Fig. 5. a) tomographic sectional image of the nucleus treated with 10^{-8}M DHT as a control. The image is displayed as in Fig. 5d. b) surface view of the distribution of AR-GFP in the nucleus of 10^{-6}M of nitrofen-treated cell; c) surface view of the chromatin structure of the same nucleus as shown in Fig. 5b; d) spatial merge of b) and c); e) tomographic sectional image of d); f) surface view of the distribution of AR-GFP in the nucleus of 10^{-6}M vinclozolin-treated cell. The image is displayed as in b).

matic hernia or anomalies in great vessels of newborns. Our findings suggest that after nuclear translocation, AR possibly locates in a specific region in the nucleus while demonstrating tight clustering depending on the agonist-induced transactivation competence.[3]

4. New Aspects of EED Actions Mediated by Estrogen Receptor

Several studies indicate that some EEDs function by binding to estrogen receptors α and β (ERα and β), which serve as ligand-inducible transcription factors. To identify EEDs that function via ERs, we first performed a luciferase assay using the expression vector of either ERα or β with a reporter plasmid carrying estrogen response elements. In this assay, a number of EEDs, such as bisphenol A and procymidone, induced transcriptional activity of ERs. Interestingly, some of the EEDs tested exhibited ER-subtype-specific transactivation. Dicyclohexyl phtalate and propizamide specifically activated ERα. On the contrary, p-t-butylphenol and 4, 4'-bihydroxybiphenyl activated ERβ but not ERα (Fig. 7).

ERα and β have A to F domains from the NH2 terminus to the COOH terminus that contain two transactivation functions, AF-1 and AF-2. AF-1, which is localized in the NH2-terminal A/B region, is believed to be constitutive in a cell- and promoter-specific

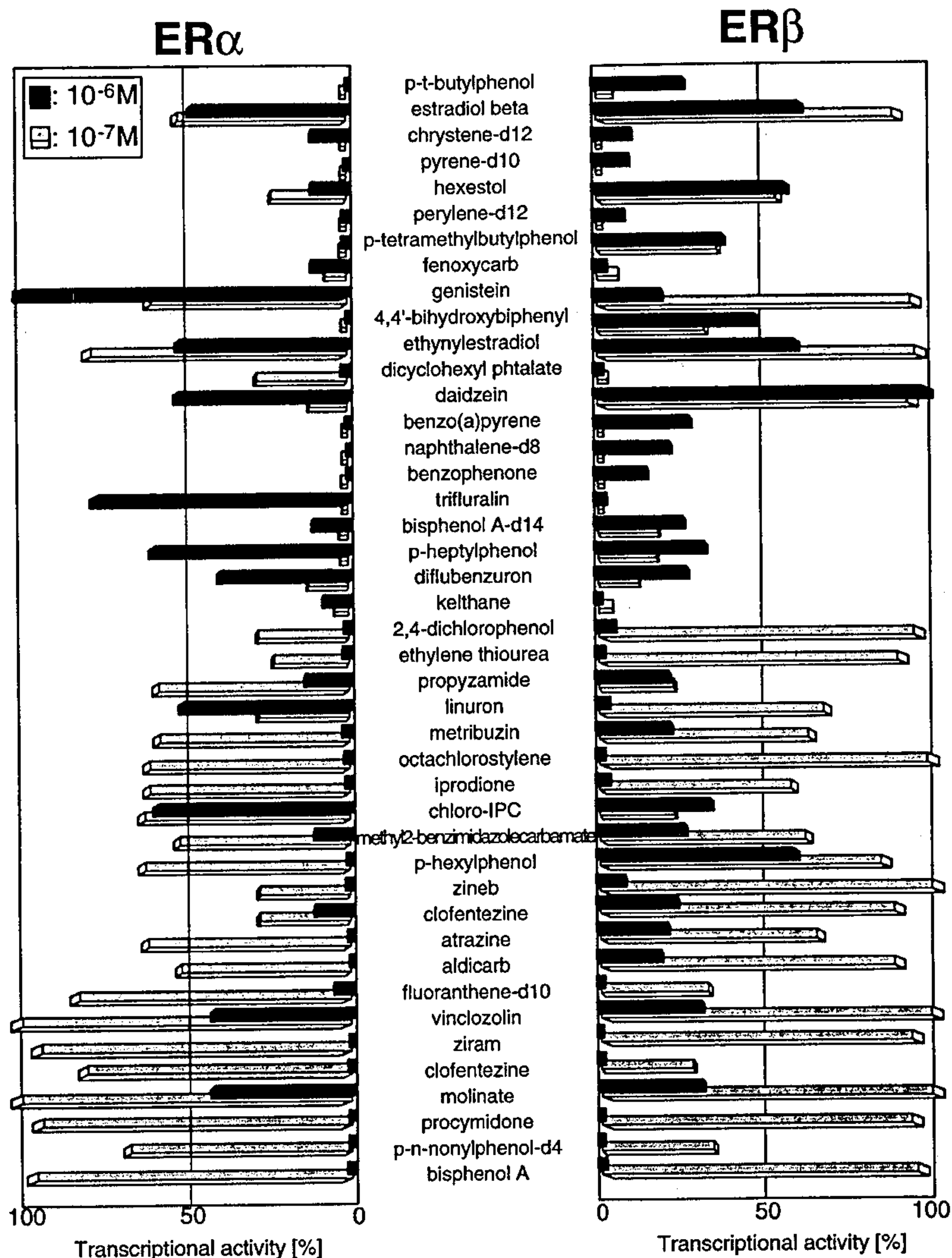

Fig. 7. Differential transactivation between ER α and β isofoms bound to EED. Relative luciferase activities are shown as compared with luciferase activities found in either ERα or β bound to bisphenol A as a control.

manner and is responsible for the partial agonist activity of tamoxifen. AF-2 residues in the COOH-terminal E region exhibits an estrogen-dependent transcriptional activity. However, the E regions of these receptors are well conserved. A/B regions have almost no homology between ERα and β. Therefore, it is thought that AF-1 is responsible for the subtype- specific activation induced by some EEDs.

The ligand-dependent activation of AF-2 requires the ligand-dependent association of coactivator complexes that contain histone acetylases, p300/CBP, P/CAF and p160 protein family members (SRC-1, TIF2, p/CIP), or non-acetylase DRIP/TRAPs. Although it is believed that AF-1 also requires coactivator complexes for its own activity, the coactivators of AF-1 have not been well characterized yet.

Therefore, we tried to identify subtype-specific coactivators of ER AF-1s using a protein purification method and yeast two-hybrid screening. Using these methods, we identified two highly related gene products, p68 and p72, which possess a DEAD -box and are thought to be putative RNA helicases.[4, 5] These proteins interact with the A/B domain of ERα but not to that of ERβ. The binding between p68/p72 and the A/B domain depends on the phosphorylation of Ser118 in the A/B domain. Furthermore, the transcriptional activity of ERα AF-1 was potentiated by p68/p72, whereas the expression of p68/p72 had no effect on either ERα AF-2, ERβ AF-2, ERβ AF-1 or other nuclear receptors tested. It was also shown that p68/p72 functioned as a large protein complex containing an RNA coactivator, SRA, p300 and p160 protein family members (SRC-1, TIF2, and p/CIP) (Fig. 8a). These findings raise the possibility that the p68/p72 coactivator complex may participate in the subtype-specific potentiation induced by endocrine disruptors (Fig. 8b).

5. Some EEDs Modify P450 Aromatase Activities in Human Granulosa-Like Tumor Cell Line, KGN

The use of tributyltin (TBT) as biocides in antifouling paints and wood preservatives results in the contamination of marine and freshwater environments. TBT has been shown to be highly toxic to a number of aquatic animals. In particular, TBT causes reproductive abnormalities and sterilization in female marine prosobranch snails. This phenomenon, which has been called either pseudohermaphroditism or imposex, is characterized by the development of additional male sex organs (penis and/or vas deferens and prostate tissue) in females. Not only TBT but also triphenyltin (TPT) has been shown to have a strong effect on the development of imposex in the rock shell, *Thais clavigera*.

We have recently established a steroidogenic human ovarian granulosa-like tumor cell line from a patient with invasive granulosa cell carcinoma.[6] The cell line possesses properties very similar to those of normal ovarian granulosa cells, including the expression of functional FSH receptor and a relatively high aromatase activity. The cell line is, thus, considered to be a very useful model for investigating the in vitro effects of various compounds on the aromatase activity in the mammalian system. To clarify the mechanism of female musculization induced by organotin compounds, we attempted to investigate whether TBT compounds have a direct effect on the aromatase activity in the mammalian system using this cell line.

The treatment with more than 1000 ng/ml TBT was very toxic to cells of the above-

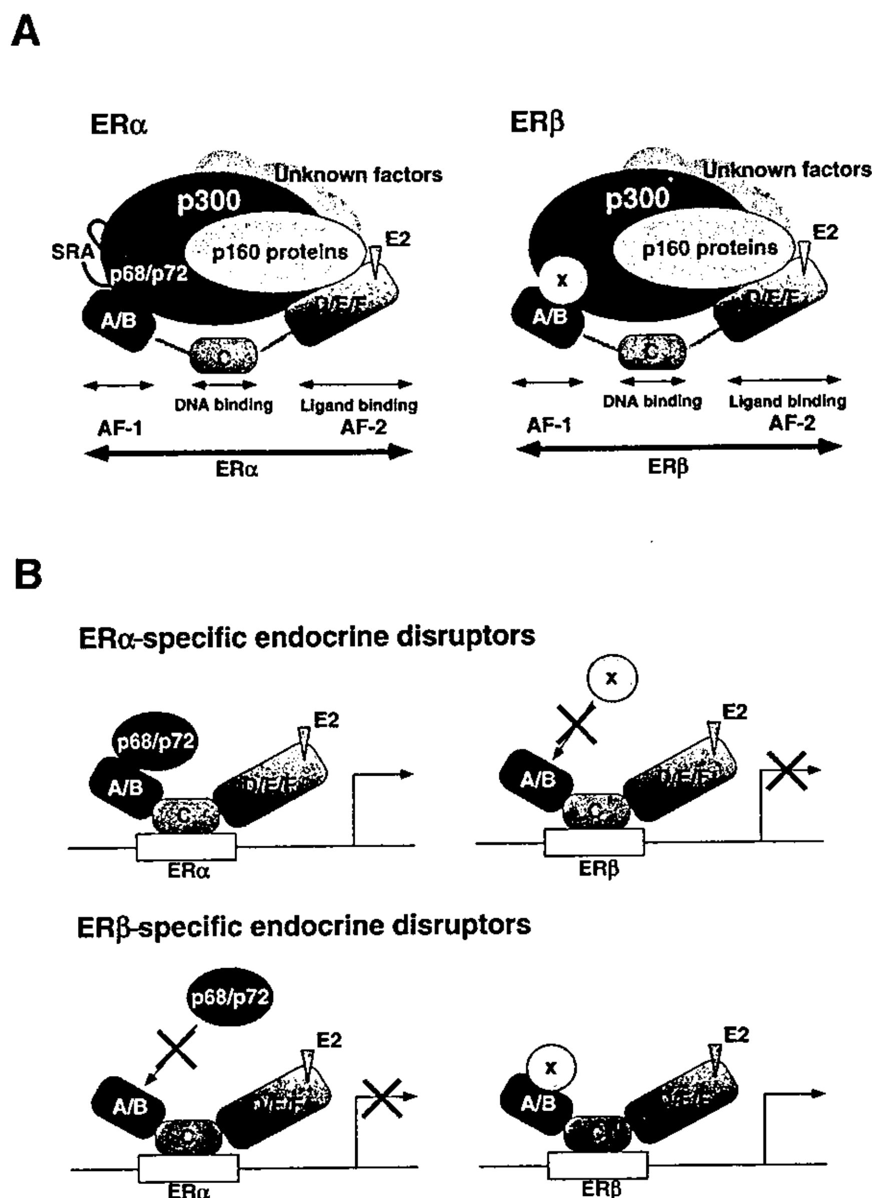

Fig. 8. A) p68/72 is an ERα A/B domain-specific coactivator. In this figure, a putative protein specifically bound to the A/B domain of ERβ is shown as "protein X". B) Hypothetical mechanisms of ER subtype-specific transactivation mediated by EED.

described cell line and caused immediate cell death within 24 h, while 200 ng/ml was found to cause apoptosis of the cells which was observed by Annexin V-EGFP/PI staining. Treatment of the KGN cells for more than 48 h with 20 ng/ml TBT or TPT, which is the concentration level reported to cause imposex in marine species, did not affect the cell proliferation but significantly suppressed the aromatase activity as determined by a $[^3H]H_20$ release assay (Fig. 9a). Treatment with 20 ng/ml TBT for 7 days also resulted in reduced E2 production from Δ4–androstenedione as stimulated by db-cAMP (Fig. 9b). The

changes in the aromatase activity induced by TBT were associated with comparable changes in the expression level of P450arom mRNA as assessed by RT-PCR. The luciferase activity of P450arom promoter II (1 kb) decreased after the addition of 20 ng/ml TBT to transfected KGN cells either in the untreated state or in states stimulated by db-

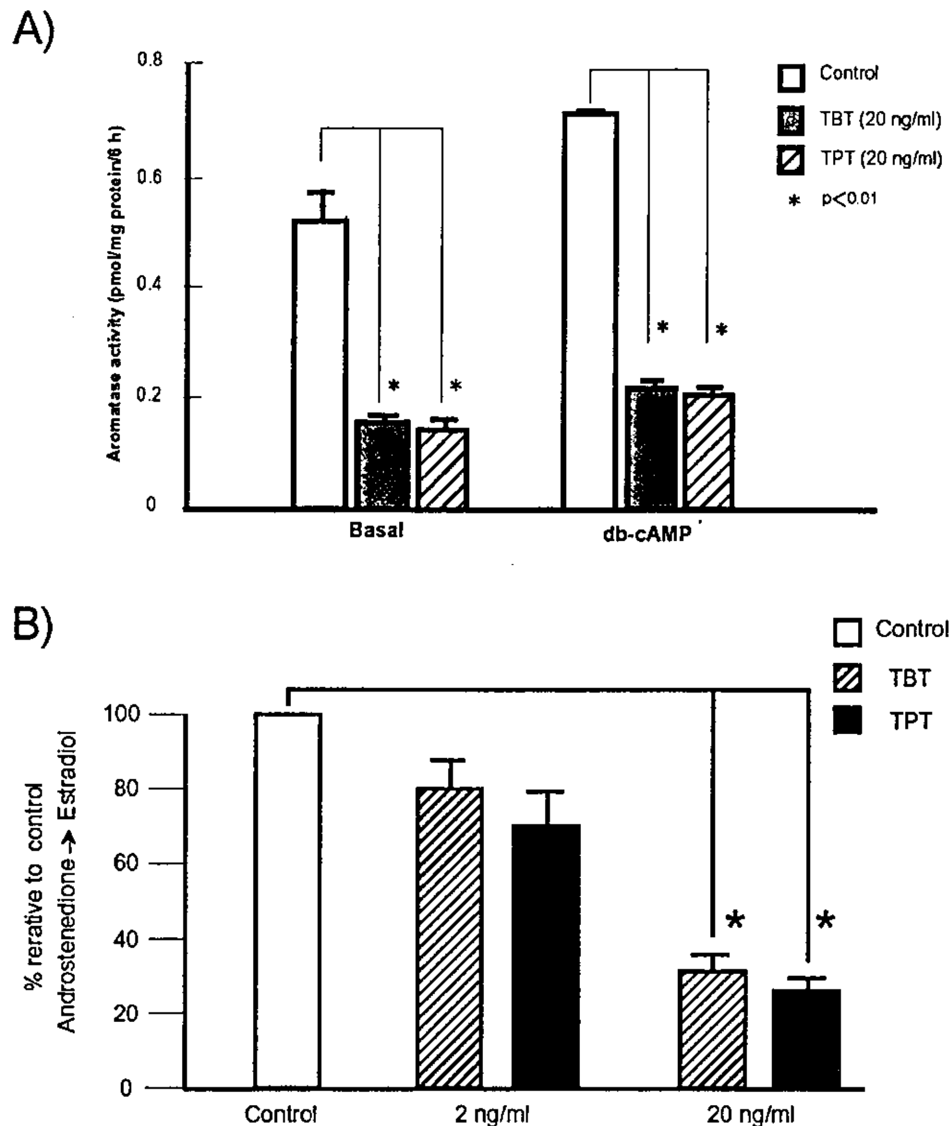

Fig. 9. a) Regulation of aromatase activity in cultured KGN cells. The granulosa cancer cells, KGN cells, were treated with 20 ng/ml TBT (black bar) or TPT (hatched bar) for 48 h in the presence or absence of 10^{-4} M db-cAMP. The data represent means ± SD from three independent experiments carried out in triplicate. Aromatase activity was standardized based on the protein concentration and is presented as pmol/mg protein/6 h. *$p < 0.01$ *vs.* control cells treated with ethanol (open bar); b) Effects of TBT and TPT on E2 production in cultured KGN cells. The cells were treated for 7 days with 2 or 20 ng/ml TBT (hatched bar) or TPT (black bar). E2 production was assayed by RIA. The data represent means ± SD from three independent experiments carried out in triplicate. *$p < 0.05$ *vs.* control cells treated with ethanol (open bar).

cAMP. The Ad4BP-dependent increase in the luciferase activity of P450arom promoter II was also downregulated by such treatments. These results indicate that TBT inhibited aromatase activity and also decreased the P450arom mRNA expression level at the transcriptional level in KGN cells.[7]

In contrast to organotin compounds, we identified benomyl as the EED which enhances aromatase activity. In KGN cells, benomyl enhances the activity as measured by $[^3H]H_20$ release assay by two to hour folds. To the best of our knowledge, this is the first EED enhancing the aromatase activity. Preliminary data suggest that the activation mechanism is, at least in part, exerted at the aromatase gene transcription level.

6. Effect of EEDs on Primordial Germ Cell Development in Mouse

Primordial germ cells (PGCs) are the origin of gametes. In the mouse embryo, a cluster of about 50 PGCs can be detected in gastrulating embryos at approximately 7.25 dpc in the extraembryonic mesoderm at the base of the amniotic fold. From this region, PGCs spread into the mesoderm of the primitive streak, and the endoderm of the yolk sac and hind gut. At 10 dpc, they begin to translocate by active migration up to the dorsal mesentery and into the gonadal ridges where, at approximately 12.5 dpc, they differentiate into oogonia/oocytes and prospermatogonia in the ovary and testis, respectively. The migration is accompanied by extensive proliferation of PGCs, and the number of PGCs dramatically increases up to several thousands by the time they reach the gonadal ridge. After populating the gonadal ridge, a reciprocal interaction between germ cells and gonadal somatic cells becomes evident and is required for full development of the gonad. Despite the important role of germ cells in gonadal development, to the best of our knowledge, the effect of EEDs on germ cell development has not been reported yet. In this study, we show for the first time the effect of EEDs on PGC development prior to gonad formation.

Taking advantage of alkaline phosphatase (AP) activity of PGCs, we visualized the PGCs in whole mount by AP staining and counted the PGCs under a microscope. PGCs stain strongly in a characteristic pattern of AP activity during their proliferation in the developing hindgut and migration into genital ridges. Vinclozolin (0.2 mg/g weight dose) was orally administered to pregnant mice daily from 5.5 to 8.5 dpc. At 9.5 dpc, the mice were sacrificed and the embryos were subjected to AP staining. The embryos from the mothers treated with vinclozolin, although indistinguishable from the wild-type control in terms of overall size and morphological features, had reduced numbers of PGCs as shown in Fig 10. PGC number determined from whole-mount embryos was plotted against somite number. The slope of the regression line for the vinclozolin-treated mice was smaller than that for the wild-type mice, suggesting the proliferation or survival of PGCs might have been affected. Since vinclozolin was found to have an anti-androgenic function in vitro, which is one of the molecular feature to be considered of as EED, it is interesting to know whether this effect on PGC differentiation can be explained by the same mechanism as anti-androgenic action. Thus, flutamide (0.2 mg/g weight dose) was administered for 3 days from 5.5 to 7.5 dpc, and the embryos were recovered at 8.5 dpc. As shown in Fig. 10, the number of PGCs was reduced. These results demonstrated for the first time that EED acting as anti-androgen hormones affect not only gonadal development and sexual differen-

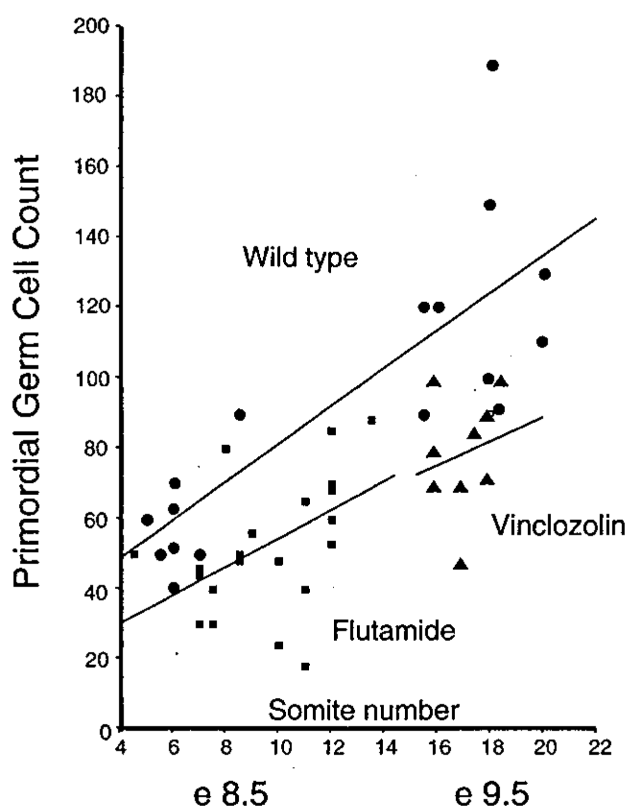

Fig. 10. Effect of EEDs on primordial germ cell development in mouse. C57/B6J pregnant mice were orally administered with chemicals as shown in the figure. At each embryonic day indicated in the figure, the mice were sacrificed, then the embryos were collected and were subjected to alkaline phosphatase staining. Filled circles, wild type mice fed with vehicle; squares, mice fed with flutamide; triangles. mice fed with vinclozolin.

tiation but also germ cell development in early embryos. Thus, exposure of EEDs in early gestational stage must be prevented.

7. Metabolism of EEDs: Implication of SXR

Steroid xenobiotics receptor (SXR) regulates the CYP3A gene expression in response to exogenous chemicals, such as EDC, after binding to motifs (SXRE) that contain either direct repeats or everted repeats separated by different numbers of spacer nucleotides. With the cotransfection of a plasmid that express the human SXR protein, we transfected, into CV-1 cells, three reporter plasmids, which harbor SXRE from either the rat or human CYP3A gene, or a Liver X Receptor binding site (LXRE) from the human CYP7A gene. The cells were treated with 55 kinds of EEDs.

We identified 14 EEDs, including benzo (A) pyrene, alachlor, nitrofen, nonylphenol, kelthane, methoxychloras, fenvalerate, octylphenol, TMBP, esfenvalerate, endrin, transnonachlor, DDT, DDD, and *p, p'*-DDE, as compounds that induce human SXR-mediated transcription (in preparation). These chemicals, as well as corticosterone, rifampicin, and troglitazone, activated all three reporter genes nearly uniformly, which is in

close agreement with the characteristics of the activated SXR. Furthermore, we constructed an expression plasmid for the chimeric SXR protein, the C- terminus of which was fused to the cyan fluorescence protein. A confocal microscopy image analysis revealed that these 14 chemicals induced the intranuclear fluorescence foci produced by the chimeric protein. We thus concluded that these chemicals are real activators of the CYP3A system linked to SXR.

Acknowledgement

In this report, we summarized the recent progress of our study on the molecular mechanisms of EED action. This work was performed under a financial support from CREST. Part of this work was performed at Kyushu University Station for Collaborative Research.

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Nuclear Receptor Function Requires a TFTC-Type Histone Acetyl Transferase Complex

Junn Yanagisawa,[1,6] Hirochika Kitagawa,[1]
Mitsuaki Yanagida,[3] Osamu Wada,[1] Satoko Ogawa,[1]
Madoka Nakagomi,[1,6] Hajime Oishi,[1]
Yasuji Yamamoto,[1] Hiromich Nagasawa,[2]
Steven B. McMahon,[4] Michael D. Cole,[4]
Laszlo Tora,[5] Nobuhiro Takahashi,[3]
and Shigeaki Kato[1,6,7]
[1]Institute of Molecular and Cellular Biosciences
[2]Department of Applied Biological Chemistry
Graduate School of Agricultural and Life Sciences
University of Tokyo
Yayoi, Bunkyo-ku, Tokyo 113-0032
[3]Department of Applied Biological Science
University of Agriculture and Technology
Saiwai-cho, Fuchuu-City, Tokyo 183-8509
Japan
[4]Department of Molecular Biology
Princeton University
Princeton, New Jersey 08544
[5]Institut de Genetique et de Biologie Moleculaire
et Cellulaire
CNRS/INSERM/ULP
BP 163, 67404 Illkirch Cedex
CU de Strasbourg
France
[6]CREST
Japan Science and Technology
4-1-8 Honcho, Kawaguchi, Saitama 332-0012
Japan

Summary

Nuclear receptors (NRs) regulate transcription in a ligand-dependent way through two types of coactivator complexes: the p160/CBP histone acetyl transferase (HAT) complex and the DRIP/TRAP/SMCC complex without HAT activity. Here we identified a large human (h) coactivator complex necessary for the estrogen receptor α (ERα) transactivation. This complex contains the GCN5 HAT, the c-Myc interacting protein TRRAP/PAF400, $TAF_{II}30$, and other subunits. Similarly to known TFTC (TBP-free TAF_{II}-containing)-type HAT complexes (hTFTC, hPCAF, and hSTAGA), TRRP directly interacted with liganded ERα, or other NRs. ERα transactivation was enhanced by the purified complex in vitro. Antisense TRRAP RNA inhibited estrogen-dependent cell growth of breast cancer cells. Thus, the isolated TFTC-type HAT complex acts as a third class of coactivator complex for NR function.

Introduction

Liphophilic ligands such as steroid/thyroid hormones and fat-soluble vitamins are thought to exert their actions through transcriptional controls of target genes by their cognate nuclear receptors (NRs). NRs form a steroid/thyroid hormone superfamily and act as a ligand-inducible transcription factor (Beato et al., 1995; Mangelsdorf et al., 1995; Chambon., 1996). From their functional and structural similarities, NR proteins are divided into six (or five) functional domains designated as A–F. The most-conserved DNA binding domain is located in the C domain, and the less-conserved C-terminal E/F domain is mapped as the ligand binding domain (LBD). The N-terminal A/B and the LBD are responsible for ligand-dependent transactivation function of NRs (Tora et al., 1989). The N-terminal activation function (AF-1) in the A/B domain is constitutively active on its own, while the AF-2 function in the LBD is induced upon ligand binding. However, the activities of both of AFs are dependent on cell type and promoter content (Tora et al., 1989; Beato et al., 1995; Watanabe et al., 2001).

NRs require coactivator complexes along with basic transcription machinery to activate transcription (Freedman, 1999; Glass and Rosenfeld, 2000), like the other activators. Two distinct classes of NR coactivator complexes have been identified to date to directly associate with and activate AF-2 of NRs. One class (designated as p160/CBP complex hereafter) contains CBP/p300 (Kamei et al., 1996; Ogryzko et al., 1996) and p160 (SRC-1/TIF2/AIB1) family proteins (Onate et al., 1995; Voegel et al., 1996; Anzick et al., 1997; Torchia et al., 1997) and the others (Yanagisawa et al., 1999; DiRenzo et al., 2000; Watanabe et al., 2001), which are supposed to modulate chromatin structure in terms of their intrinsic histone acetyltransferase (HAT) activities and the action of the associated chromatin-remodeling proteins. The other class is a non-HAT coactivator complex, DRIP/TRAP/SMCC (Fondell et al., 1996; Gu et al., 1999; Ito et al., 1999; Rachez et al., 1999). Both p160/CBP and DRIP/TRAP complexes look equally potent with regard to activating the function of most liganded NRs in vitro, while their functional difference is suggested by the findings that the two complexes are sequentially recruited from the p160/CBP complex to the DRIP/TRAP complex to liganded NRs bound upon the target gene promoters (Shang et al., 2000). Moreover, in mice, lack of ligand response in target tissues was observed in only limited, but not in all, members of NRs by gene disruption of major components in these complexes (Xu et al., 1998; Ito et al., 2000). Considered together with the cell type- and promoter-content-specific properties of the AFs, a possibility is raised that the other unknown coactivator complexes and/or novel factors in the known complexes support transactivation function of NRs.

To address this issue, we purified HAT complexes associated with liganded estrogen receptor α (ERα) LBD from the HeLa cell nuclear extract. One of purified, large multiprotein complexes contained GCN5 HAT (Wang et al., 1997), the c-Myc-interacting protein TRRAP/PAF400 (McMahon et al., 1998; Ogryzko et al., 1998; Vassilev et al., 1998), and TAFII30 (Jacq et al., 1994), which are common factors shared with hTFTC, hPCAF, and hSTAGA HAT coactivator complexes (Martinez et al., 1998; Ogryzko et al., 1998; Wieczorek et al., 1998; Brand

[7]Correspondence: uskato@mail.ecc.u-tokyo.ac.jp

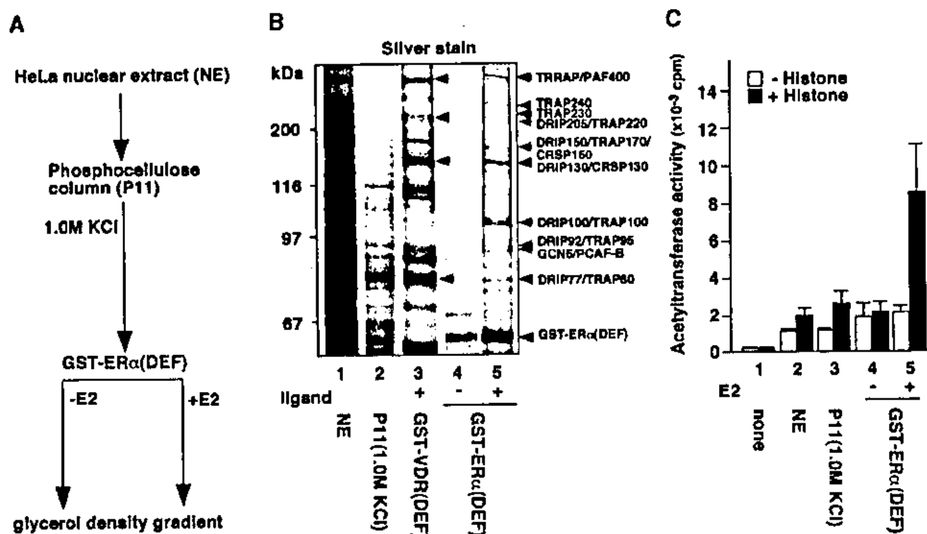

Figure 1. Purification and Identification of Proteins Interacting with E_2-Bound ERα

(A) Purification schema for E_2-bound ERα-interacting proteins. Nuclear extracts prepared from HeLa S3 cells were applied to a P11 phosphocellulose column. After extensive washing with wash buffer containing 0.15 M KCl, bound proteins were eluted with wash buffer containing 1.0 M KCl. Eluted fractions were then incubated with immobilized GST-ERα(DEF) in the presence or absence of E_2 (10^{-6} M). ERα-interacting proteins were eluted from the GST-ERα(DEF) column by N-lauroyl sarkosine.

(B) Identification of ligand-dependent ERα(DEF)-interacting proteins. A panel of fractions, as indicated, was subjected to SDS-PAGE followed by silver staining. Total HeLa S3 nuclear extract (lane 1), fraction eluted from P11 column [P11(elute)] (lane 2), the eluted fractions from liganded-GST-VDR(DEF) column [GST-VDR(DEF) + 1,25(OH)$_2$D$_3$] (lane3), and unliganded- and liganded-GST-ERα(DEF) columns [GST-ERα(DEF) − E2; GST-ERα(DEF) + E2] (lanes 4 and 5) are shown. Proteins eluted from the GST-ERα(DEF) column (lane 4) were examined by mass spectrometry and identified proteins indicated at the right side of the panel.

(C) Histone acetyltransferase (HAT) activity in purified fractions. The indicated fractions were incubated with either free histones (closed bars) or BSA (open bars), together with ^{3}H-labeled acetyl-CoA, and assayed for acetyltransferase activity (HAT) in a filter binding assay (Rachez et al., 1998). HAT activity is quantitated as radioactivity (cpm) of ^{3}H-labeled acetyled histones.

et al., 1999a, 1999b). Three LXXLL motifs mapped in the middle of TRRAP served as a direct and ligand-dependent surface for ERα and other NRs. Coexpression of TRRAP and GCN5 cooperatively enhanced the ligand-induced transactivation function in vivo, and the ERα transactivation function was potentiated by the purified complex in vitro. A chromatin immunoprecipitation (ChIP) analysis revealed that the TRRAP/GCN5 complex is recruited in an estrogen-dependent manner to the target gene promoters for ERs. Thus, the present study suggests that the purified TFTC-type HAT complex functions as a third class of NR coactivator complexes.

Results and Discussion

Purification of a Multiprotein Complex Associated with Liganded ERα AF-2 Domain

To identify coactivator complexes for ERα, a HeLa cell nuclear extract-derived fraction, which was prepurified on a phosphocellulose column (P11) (see Experimental Procedures), was incubated with a glutathione-S-transferase (GST)-fused LBD of ERα in the presence or absence of 17β-estrogen (E2), and found that the 1.0 M KCl elute fraction from the P11 column contains abundant amounts of putative complexes (Figure 1A). Proteins that interacted with ERα LBD were separated by SDS-PAGE and silver stained (Figure 1B). A number of proteins bound the ERα LBD in a ligand-dependent way (Figure 1B, compare lanes 4 and 5), and interestingly, some of them were not detected by purification on the liganded LBD of the vitamin D receptor (VDR) using the same purification procedure (Figure 1B, lane 3). As HAT activities of p160/CBP and other coactivator complexes are considered to acetylate nucleosomes to facilitate transcription initiation (Kuo and Allis, 1998), we measured whether proteins binding to ERα LBD had HAT activity (Rachez et al., 1998) (Figure 1C). ERα-interacting proteins dissociated from the liganded GST-ERα LBD exhibited significantly higher HAT activity than those eluted either from the nonliganded ERα LBD or the phosphocellulose column (Figure 1C, compare lanes 3 and 4 to lane 5). To identify the proteins that bound to the liganded ERα LBD, we performed the peptide mass fingerprinting of polypeptides specifically bound to the liganded ERα LBD. The obtained masses and the apparent molecular weight of the different polypeptides revealed that the fraction eluted from the liganded ERα LBD contained the GCN5 HAT (Wang et al., 1997) together with the c-Myc interacting protein TRRAP/PAF400 (McMahon et al., 1998; Ogryzko et al., 1998; Vassilev et al., 1998), suggesting that protein complexes containing these factors bind specifically to the liganded ERα LBD. Moreover, in agreement with previous results (Fondell et al., 1996; Gu et al., 1999; Ito et al., 1999; Rachez et al., 1999), several components of the DRIP/TRAP/SMCC complex were also identified from this fraction and the liganded VDR LBD-bound fraction (Figure 1B, lanes 3 and 5). In contrast, the p160 family of

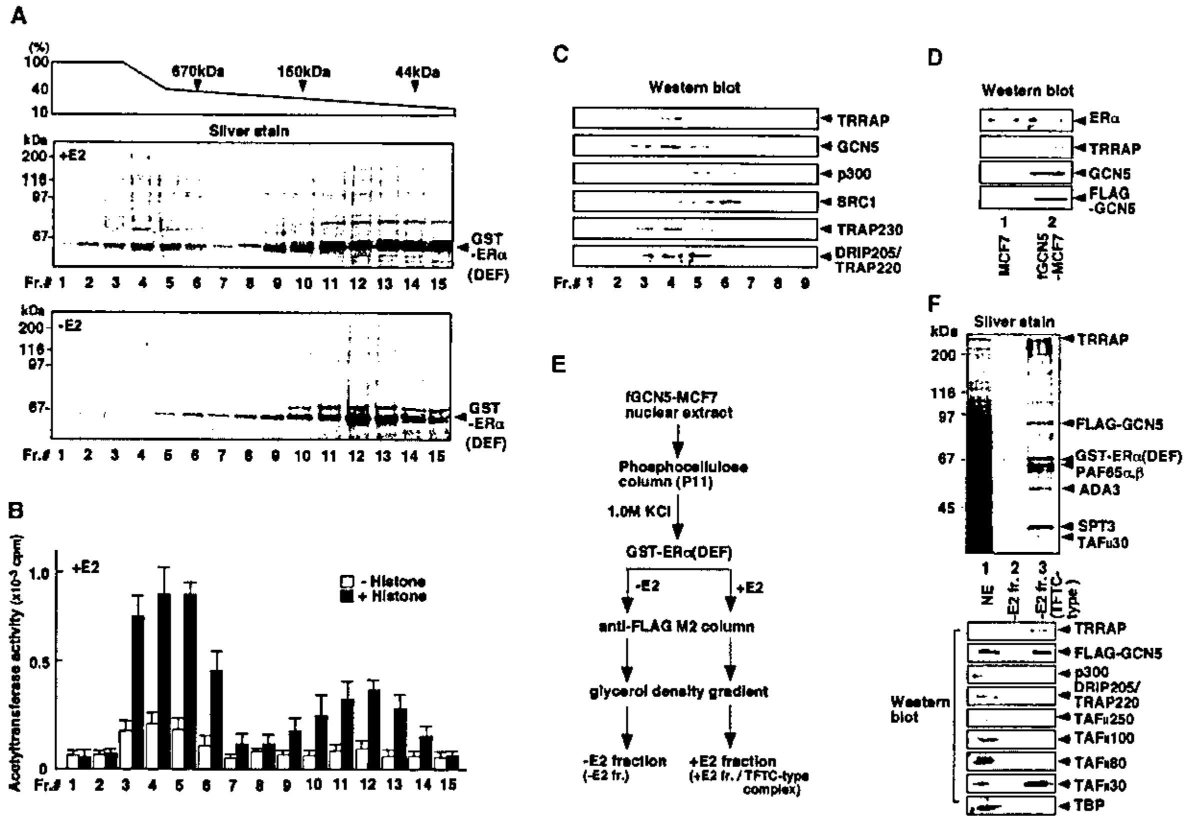

Figure 2. A TRRAP/GCN5 Complex Interacts with ERα(DEF) in a Ligand-Dependent Manner

(A) Glycerol gradient analysis. Eluted fractions from a P11 column were passed over an immobilized GST-ERα(DEF) column in the absence or presence of 10^{-8} M E_2. Protein complexes associated with GST-ERα(DEF) were then dissociated from the reduced glutathione and applied to 10%–40% glycerol gradients. Upper panel, SDS-PAGE analysis of glycerol gradient fractions of GST-ERα(DEF)-interacting proteins in the presence of E_2 (10^{-8} M). Lower panel, fractions of GST-ERα(DEF)-interacting proteins in the absence of E_2. The positions of marker proteins of known molecular masses are shown above the panels.

(B) HAT activity in glycerol gradient fractions. HAT activity in each gradient fraction was quantitated in the presence (closed bars) or absence (open bars) of histones.

(C) Western blot analysis of glycerol gradient fractions. To identify proteins contained in each gradient fraction, Western blot analysis was performed using specific antibodies against TRRAP, GCN5, p300, SRC-1, TRAP230, and DRIP205/TRAP220.

(D) Ectopic expression of FLAG-tagged GCN5 in MCF7 stable transformants. Expression of exogenous FLAG-GCN5 and endogenous TRRAP and ERα proteins in wild-type MCF7 (lane 1) or MCF7 cell line constitutively expressing FLAG-GCN5 (fGCN5-MCF7) (lane 2) were tested by Western blot analysis using antibodies against FLAG, TRRAP, and ERα.

(E) Purification schema of GCN5 complexes from MCF7 stable transformants. ERα-interacting complexes were purified from nuclear extracts of MCF7 stable transformants ectopically expressing FLAG-GCN5 using phosphocellulose P11 and GST-ERα(DEF) columns. Protein complexes containing GST-ERα(DEF) were eluted from glutathione beads with reduced glutathione and then applied to anti-FLAG affinity columns. Protein complexes containing FLAG-GCN5 were then eluted from anti-FLAG affinity resin by FLAG peptide for further analyses.

(F) Ligand-dependent interaction of TRRAP/GCN5 complexes with ERα. Fractions eluted from anti-FLAG affinity resin in the presence of E_2 (+E_2 fraction) and absence of E_2 (−E_2 fraction) were subjected to SDS-PAGE followed by silver staining. Separated proteins were further identified by both mass spectrometric (upper panel) and Western blot (lower panel) analyses using antibodies as indicated at the right side of the panel.

proteins, CBP/p300 or p68/p72 (Endoh et al., 1999; Watanabe et al., 2001) was not identified from this liganded ERα LBD-bound fraction by mass spectrometry, presumably due to their low abundance in the prepurified fraction.

To test whether a GCN5-type HAT complex or other HAT complexes contribute to the measured HAT activity in the ERα-bound fraction, the ERα-interacting proteins were further fractionated according to their molecular masses by a glycerol gradient (Figure 2A). The HAT activity in each fraction was then measured (Figure 2B). In parallel, proteins in each fraction were separated by SDS-PAGE and silver stained (Figure 2A), or analyzed by Western blot using specific antibodies against TRRAP, GCN5, components of the p160/CBP complex (p300, SRC-1), and components of the DRIP/TRAP complex (DRIP205/TRAP220, TRAP230) (Figure 2C). In glycerol gradient fractions 3 to 6, where liganded ERα LBD-bound proteins were separated, protein complexes with a molecular mass larger than 670 kDa were clearly visible (Figure 2A, upper panel), whereas these complexes were absent in the control fractions where proteins were eluted from the nonliganded ERα LBD (Figure 2A, lower panel). HAT assays revealed that fractions containing

multiprotein complexes with more than 670 kDa possess high HAT activities (Figure 2B). Western blot analysis revealed the presence of GCN5 HAT together with TRRAP, DRIP205/TRAP220 in fractions 3 and 4 (Figure 2C), while other HATs, such as p300 and SRC-1, were detected in fractions 5 and 6 (Figure 2C). Thus, our results indicate that an endogenous human GCN5/TRRAP HAT-containing complex binds liganded ERα, together with previously identified coactivators (such as the DRIP/TRAP/SMCC complex, p300 and SRC1).

Direct and Ligand-Dependent Interaction of ERα AF-2 Domain with a TFTC-Type HAT Complex through Three LXXLL Motifs in TRRAP

To better characterize and identify other components of the GCN5/TRRAP complex that bind to the liganded ERα LBD, we generated a stable MCF-7 human breast cancer cell line expressing FLAG-tagged GCN5, since GCN5 and TRRAP are common components shared with hTFTC (McMahon et al., 1998; Wieczorek et al., 1998; Brand et al., 1999a, 1999b), hPCAF (Ogryzko et al., 1998), and hSTAGA (Martinez et al., 1998) complexes to form a class of coactivator complexes with slightly different subunit compositions. An expression of FLAG-GCN5 in the selected clone (fGCN5-MCF7) was detected by Western blot analysis using an antibody against the FLAG epitope (Figure 2D). The ERα-bound complexes containing FLAG-GCN5 were then purified according to the same scheme as before (Figure 2E). In this complex, FLAG-GCN5 was copurified together with TRRAP and a 30 kDa TAF_{II} ($hTAF_{II}30$) (Jacq et al., 1994), whereas p300, DRIP205/TRAP220, other $hTAF_{II}$s ($TAF_{II}250$, $TAF_{II}100$, and $TAF_{II}80$), and hTBP were undetectable by Western blot analysis (Figure 2F, lower panel). Protein identification of the purified proteins by mass-spectrometric analysis identified TRRAP, GCN5, PAF65α, β, hADA3, hSPT3, and $hTAF_{II}30$ (Figure 2F, upper panel), which are all common components of TFTC and PCAF complexes (Ogryzko et al., 1998; Wieczorek et al., 1998; Brand et al., 1999b). These findings suggest that the complex purified from MCF7 cells belongs to a class of the known GCN5 HAT-containing TFTC-type complexes and that these complexes may have a coactivator activity for NRs.

Ligand-dependent interactions of NRs with their coactivators are known to be mediated through direct contacts between LXXLL motif(s) in the coactivators and the most C-terminal α-helix (helix12) of the NR LBD (Heery et al., 1997; Voegel et al., 1998; Glass and Rosenfeld, 2000). To identify directly interacting components for ERα in this TFTC-like complex, we searched the amino acid sequences in all of the known components of TFTC, hSTAGA, and PCAF/GCN5 and found that TRRAP contains ten LXXLL motifs in its entire sequence. The fact that the yeast homolog of TRRAP, Tra1, participates in direct activator interactions critical for transcription activation (Brown et al., 2001), together with the finding that TRRAP was abundant in our purification process, further suggested that TRRAP is the factor that recruits the purified TFTC-like complex to ERα. Thus, we analyzed the direct and ligand-dependent interaction of TRRAP with ERα by a GST pull-down assay. The full length of TRRAP exhibited direct and ligand-dependent interaction with ERα, and the region responsible for this interaction was mapped in between amino acids 984–1214 of TRRAP, harboring three LXXLL motifs (Figure 3A). A series of mutations in each of the three motifs that introduce amino acid substitution (Figure 3B) demonstrated that all of the three motifs contribute the efficient interaction with E2-bound ERα (Figure 3B). Importantly, ligand-dependent interactions were observed also with other NRs, such as in ERβ, VDR, and PPARγ (Figure 3C), as expected from the property of the LXXLL motif (Heery et al., 1997; Voegel et al., 1998). We then tested by immunoprecipitation whether the direct and ligand-dependent interaction between ERα and TRRAP takes place also in the cells. Endogenous ERα was immunoprecipitated from MCF-7 cells grown in the absence and presence of 17β-estradiol and tested for the presence of TRRAP and other subunits (Figure 3D). Western blot analysis showed that both endogenous TRRAP and GCN5 are coprecipitated with ERα in an E2-dependent manner (Figure 3D). Thus, the endogenous ERα is able to stably associate with the endogenous GCN5/TRRAP HAT complex. To further study the association of the GCN5/TRRAP HAT complex and ERα, the purified FLAG-GCN5-containing complex was disrupted by sonification and then incubated with GST- ERα LBD in the absence or presence of E2. A significant retention of TRRAP by the liganded ERα was found by Western blotting and mass-spectrometric analysis (Figure 3E), indicating again that TRRAP serves as a direct surface in the TRRAP/GCN5 complex for ligand-dependent interactions with ERα and the other NRs. This is in agreement with the recent finding that Tra1, the yeast homolog of TRRAP, serves as a common and direct target for acidic activators (Brown et al., 2001).

Potentiation of Nuclear Receptor Transactivation Function by the TFTC-Type Complex

To investigate whether the TRRAP/GCN5 complex acts indeed as a coactivator complex of ERα, a cell-free in vitro transcription assay was performed with the purified FLAG-GCN5 complex as prepared for mass-spectromic analysis (Figure 2E). The chimeric protein of the ERα AF-2 LBD domain fused to the GAL4 DNA binding domain was expressed as a GST-fusion protein, purified, and applied to this assay with a G-less cassette reporter plasmid bearing five GAL4-DNA binding sites (17 × 5 m). The addition of the purified GCN5/TRRAP HAT complex to the rat liver nuclear extract increased the activation of the liganded ERα 3- to 4-fold when compared with the activation obtained with the liganded ERα alone (Figure 4A, compare lanes 4 and 5 to 6 and 7). Moreover, in a transient expression assay using a luciferase reporter driven by an estrogen response element (ERE)-containing promoter, coexpression of either TRRAP or GCN5 enhanced the E2-induced transactivation of ERα (Figure 4B, lanes 2 and 3). We obtained a comparable enhancement of activation with the previously described cofactor TIF2 (Voegel et al., 1996) (Figure 4B, lane 6), while DRIP205/TRAP220 was not very potent in this assay (lane 5). Ligand-dependent transactivation of other NRs (such as ERβ, VDR, and PPARγ) was also enhanced by expression of either TRRAP or GCN5 (Figure 4B, lanes 7–15), in agreement with the previous report that hTRβ transactivation function is enhanced by

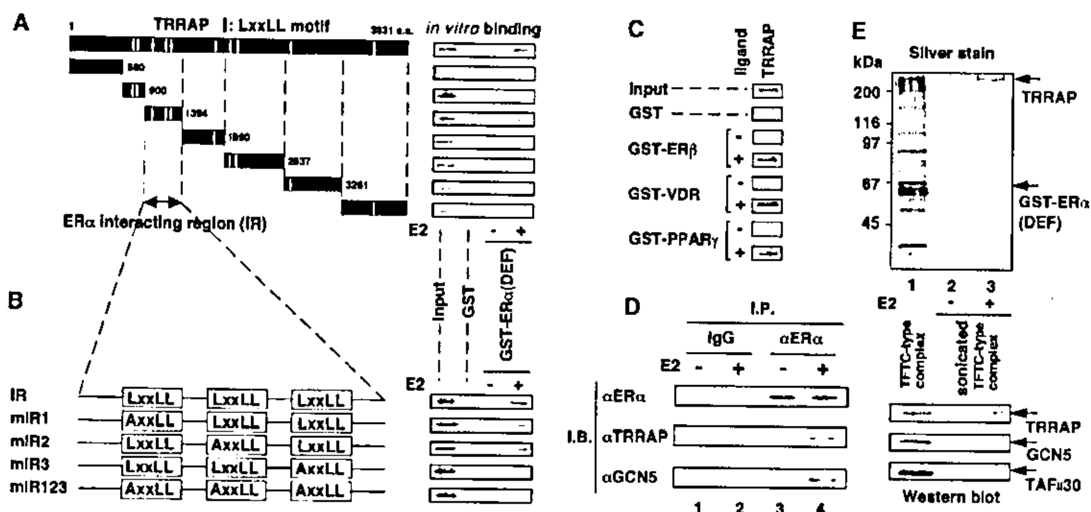

Figure 3. Ligand-Dependent Interaction of a TFTC-Type TRRAP/GCN5 Complex with ERα via LXXLL Motifs in TRRAP

(A) Direct, ligand-dependent interaction of TRRAP with ERα in vitro. To map the ERα-interacting region of TRRAP protein, deletion mutants of TRRAP were translated in vitro and incubated with either GST or GST-ERα(DEF) immobilized on glutathione-Sepharose beads in the presence or absence of 10^{-8} M E_2. Bound proteins were subjected to SDS-PAGE followed by autoradiography.

(B) The three LXXLL motifs in the ERα-interacting region of TRRAP are essential for E_2-dependent interaction. A series of mutants with amino acid replacements in the LXXLL motifs were examined for ligand-dependent interaction with ERα.

(C) Ligand-dependent interaction of TRRAP with other NRs. In vitro-translated full-length TRRAP protein was incubated with GST-fused LBD of the indicated NRs in the presence or absence of cognate ligand (10^{-6} M). Bound proteins were subjected to SDS-PAGE followed by autoradiography.

(D) E_2-dependent interaction between ERα and TRRAP/GCN5 complexes in vivo. MCF-7 cells were lysed and subjected to immunoprecipitation using mouse anti-ERα or normal mouse immunoglobulin as a control in the presence or absence of E_2 (10^{-8} M). Precipitates were Western blotted with antibodies to ERα, GCN5, or TRRAP as indicated.

(E) Direct, E_2-dependent interaction between ERα and TRRAP. Purified TRRAP/GCN5 complex was disrupted by sonication and incubated with immobilized GST-ERα(DEF) resin in the presence or absence of E_2 (10^{-8} M). Bound proteins were separated by SDS-PAGE and identified by mass spectrometry (upper panel) and Western blot (lower panel).

GCN5 (Anafi et al., 2000). Coexpression of TRRAP together with GCN5 additively enhanced the ligand-induced transactivation of ERα (Figure 4B, lane 4), further supporting the idea that the identified TFTC-type complex serves as a coactivator complex during the ERα activation process. Furthermore, transactivation functions of c-Myc (Kretzner et al., 1992) and VP16 were potentiated by expressions of TRRAP and GCN5 (Figure 4B, lanes 17 and 18), in agreement with the previous findings (McMahon et al., 1998; Brown et al., 2001).

To investigate whether the TFTC-type complex is indeed recruited to liganded ERα similarly to the other known coactivator complexes in the nuclei of living cells, we performed a ChIP assay with the promoters of estrogen-responsive endogenous target genes. In agreement with the idea that a TFTC-type complex plays a coactivator role, after estrogen treatment, ERα was recruited together with GCN5 and TRRAP to the promoter regions of the cathepsin D and *c-fos* genes, harboring EREs, within 30 min after E2 stimulation (Figures 4C and 4D). Interestingly, GCN5 and TRRAP were recruited to the cathepsin D promoter slightly after the recruitment of p300 and TIF2 by the liganded ERα but appeared synchronous with that of the DRIP205/TRAP220 complex (Figure 4D). These results suggest that the TFTC-type HAT complex is recruited to the ERα-stimulated promoters after the p300 HAT complex and about the same time as the DRIP/TRAP complex. Thus, it seems that the first cofactor that acts upon ERα binding to modify the chromatin is p300/CBP (Figure 4D) and that the DRIP/TRAP and the TFTC-type complex are recruited subsequently, at about the same time, to the ERα to further modify the chromatin and to facilitate transcription initiation.

Finally, in order to test whether the direct interaction observed between TRRAP and ERα plays a biological role in the estrogen actions, the function of TRRAP was examined in the estrogen-dependent cell growth of MCF-7 breast cancer cell lines by reducing its endogenous expression level, since both of TRRAP and GCN5 are major components in a complex required for c-Myc-mediated transformation (McMahon et al., 1998; Park et al., 2001). To this end, an antisense expression vector was generated by inserting the TRRAP cDNA into the CMV expression vector in the antisense orientation (AS-TRRAP). In agreement with our above described results, the ligand-dependent transactivation function of ERα was reduced by the transient expression assay when cotransfected with AS-TRRAP (Figure 5A, lane 2). We then established several stable cell lines constitutively expressing antisense RNA for TRRAP in MCF7 cells (AS-TRRAP-MCF7). In AS-TRRAP-MCF7 cells, decreased level of the TRRAP protein was observed, whereas expression levels of the other coactivators and ERα were unchanged when compared with the parental MCF-7 cells (Figure 5B). In agreement with the above results showing that GCN5 and TRRAP are both subunits of a TFTC-type coactivator complex in the AS-TRRAP-MCF7

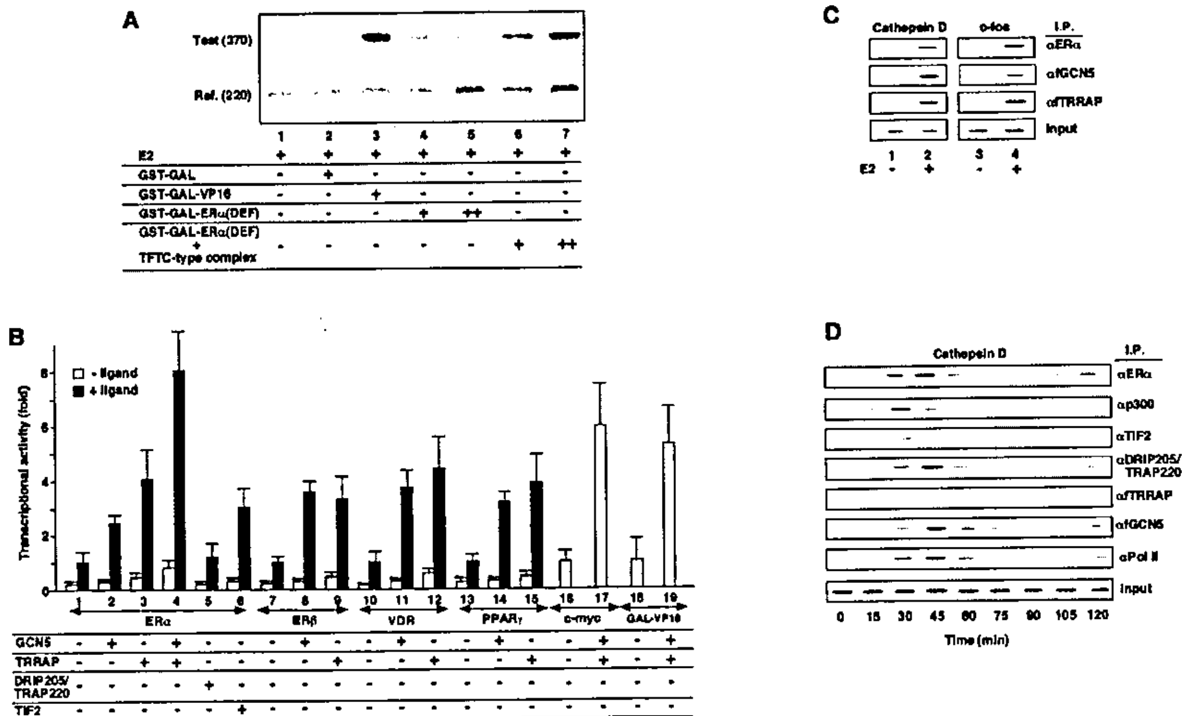

Figure 4. A TFTC-Type TRRAP/GCN5 Complex Acts as an ERα Coactivator

(A) Purified TRRAP/GCN5 complex potentiated the transactivation function of ERα by in vitro transcription. A TFTC-type TRRAP/GCN5 complex was purified according to the schema shown in Figure 2. GST-GAL (25 ng), GST-GAL-VP16 (25 ng), GST-GAL-ERα(DEF) (25[+], 50[++] ng) or GST-GAL-ERα(DEF) (25 [+], 50[++] ng) along with TRRAP/GCN5 complex was added to a 25 μl reaction mixture containing nuclear extracts in the presence of E_2 (10^{-8} M) and tested for their ability to potentiate the transactivation function of ERα by the in vitro transcription of a reporter plasmid. Reaction mixtures were incubated for 45 min and radiolabeled transcripts visualized on 6% denaturing polyacrylamide gels.

(B) Enhancement of ligand-induced transactivation function of ERα and other NRs by GCN5 and TRRAP in a transient expression assay. COS-1 cells were transfected with the expression vector of ERs (0.5 μg), GAL4DBD-VDR(DEF) (0.5 μg), GAL4DBD-PPAR(DEF) (0.5 μg), pGL-ERE-AdML (1.0 μg), and pML-CMV (10 ng) along with either pcDNA-TRRAP (1.0 μg), pcDNA-GCN5 (1.0 μg), or both in the presence or absence of cognate ligand (10^{-8} M), and the cell extracts used in a luciferase assay. Likewise, the expression vector of c-Myc (0.5 μg) and GAL-VP16 (0.1 μg) along with their reporter plasmids (M4-luc for c-Myc) (Kretzner et al., 1992) were transfected.

(C) Recruitment of ERα and GCN5 to the promoters of estrogen-responsive genes. Soluble chromatin was prepared from MCF-7 cells treated with E_2 (10^{-8} M) for 45 min and immunoprecipitated with antibodies against ERα or FLAG peptide (ChIP assay). Final DNA extractions were amplified using primer pairs that covered the cathepsin D and *c-fos* gene promoters as indicated.

(D) Dynamics of TRRAP/GCN5 complex assembly. Occupancy of the cathepsin D promoter by ERα, different coactivators, and RNA polymerase II (Pol II) at different times as measured by ChIP assay.

clones, not only the TRRAP recruitment, but also that of GCN5, was abolished to the promoter following estrogen treatment, whereas recruitment of the other coactivators was unchanged (Figure 5C). Furthermore, Northern blot analysis showed that the E2-induced expression of the endogenous *c-fos* gene was reduced in the stable cells when compared to the nontransformed MCF-7 cells (Figure 5D), indicating that in the absence of the TFTC-type HAT complex endogenous ERα cannot fully activate endogenous target genes. We next evaluated the growth rate of these stable clones. In the absence of E2, the stable transformants (AS-TRRAP-MCF7) exhibited normal cell growth similarly to the wild-type MCF-7 cells, as previously reported (McMahon et al., 1998) (Figure 5E, left panel). E2 treatments enhanced the growth rate of the wild-type MCF-7 cells (Figure 5E, right panel); however, no significant growth stimulation by E2 was observed in the stable transformants (Figure 5E, right panel). Importantly, reduction in the E2-dependent growth was observed in all of five independent clones (data not shown).

A TFTC-Type Complex Acts as a Third Class of Coactivator Complex for Nuclear Receptors

The GCN5 HAT-containing multiprotein complex isolated in this study appears to belong to the previously described TBP-free TAF_{II}-HAT (TFTC, PCAF, and hSTAGA) complexes (Ogryzko et al., 1998; McMahon et al., 1998; Martinez et al., 1998; Wieczorek et al., 1998; Brand et al., 1999b), since it contains a number of common components. The endogenous GCN5 HAT-containing multiprotein complex isolated from HeLa cells seems to have a molecular mass of about 1.5–2 MDa (Figures 2A–2C, fractions 3 and 4) and contains TRRAP in addition to GCN5. At present, due to the low abundance of this TFTC-type complex, we do not know which are the other TFTC subunits present in this complex. However, when FLAG-GCN5 was exogenously overexpressed in MCF7 cells, we isolated a multiprotein complex that is very similar to the GCN5/PCAF complex that was isolated from HeLa cells by a similar approach (Ogryzko et al., 1998), since our FLAG-GCN5 complex contains TRRAP/PAF400, PAF65β, ADA3, SPT3, and $TAF_{II}30$. Importantly,