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Human Estrogen Receptor α와 Co-activator로 구성된 바이오센서를 이용한 내분비계장애물질의 검출

Improvement of the Biosensor for Detection of Endocrine Disruptors by Combination of Human Estrogen Receptorα and Co-Activator

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Abstract

To improve sensitivity of biosensor as yeast two-hybrid detection system for estrogenic activity of suspected chemicals, we tested effects of several combinations of the bait and fish components in the two-hybrid system on *Saccharomyces cerevisiae* inducted a chromosome-integrated *lacZ* reporter gene that was under the control of *CYC1* promoter and the upstream Gal4p-binding element UAS_{GAL}. The bait components that were fused with the Gal4p DNA binding domain are full-length human estrogen receptor α and its ligand-binding domain. The fish components that were fused with the Gal4p transcriptional activation domain were nuclear receptor-binding domains of co-activators SRC1 and TIF2. We found that the combination of the full-length human estrogen receptor α with the nuclear receptor-binding domain of co-activator SRC1 was most effective for the estrogen-dependent induction of reporter activity among the two-hybrid systems so far reported. The relative strength of transcriptional activation by representative natural and xenobiotic chemicals was well correlated with their estrogenic potency that had been reported with other assay systems.

Key words: endocrine disruptors, biosensor, yeast two-hybrid system, hERα, co-activator
주제어: 내분비계장애물질, 바이오센서, 효모 이중-하이브리드 시스템, hERα, 공역인자

1. Introduction

Endocrine disruptors (EDs) that are capable of disrupting normal function of the endocrine systems through various mechanisms may pose a threat to health of human and wild lives (Colborn et al., 1993). One group of EDs is supposed to modulate function of nuclear

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receptors (NRs) by binding their ligand-binding domain. Such EDs include chemicals that are able to bind estrogen receptors (ERs), act as either agonist or antagonist. The estrogen-bound ERs regulate their target gene transcription. The ER is a member of nuclear hormone receptor superfamily (Mangelsdorf et al., 1995; Lee et al., 2003) and is composed of six functionally and physically discriminated regions (A-F). They are required for DNA binding (region C), for nuclear localization (region D), and for steroid binding (region E) (Tsai et al., 1994). In addition, there are two domains, AF-1 and AF-2, which are essential for transcriptional activation. AF-1 is located in the N-terminal region A/B and is regulated by phosphorylation in response to growth factors (Kato et al., 1995). AF-2 is located in the region E and is closely associated with the estrogen-binding domain. The activities AF-1 and AF-2 of the ER vary depending upon the responsive promoter (Metzger. et al., 1992) and cell type, and in some cases both AF domains are required for full transcriptional activation of target genes (Tzukerman et al., 1994).

The conformational change in the NR ligand-binding domain (LBD) in response to binding of hormone is responsible for recruitment of co-activators that are required for transcriptional activation by NRs (Chen and Li, 1998; McKenna et al., 1999). Steroid receptor co-activator-1 (SRC-1) is an NR co-activator sharing sequence homology with SRC-2/TIF-2 and SRC-3/p/CIP/AIB-1/TRAM-1/RAC-3/ACTR as a member of the p160 co-activator family. The p160 proteins contain a short conserved NRs interaction motif (NRs Box), which has the core sequence LXXLL (L, leucine and X, any amino acid). All of the p160s have a core NRs interaction domain that contains three NRs Boxes arranged in tandem. The LXXLL motifs in the NRs Boxes were essential for the interaction of the co-activators with the NRs (Torchia et al., 1997; Glass et al., 1997; Lee et al., 2003). The mammalian steroid hormone receptors were also shown to interact with the co-activators in a steroid-dependent manner, when introduced into *S. cerevisiae* (Ohashi et al., 1991; Purvis et al., 1991) and the yeast two-hybrid systems for the detection of endocrine disruptors have been developed so far (Nishikawa et al., 1999; Sheeler et al., 2000; Lee and Cho, 2003).

We previously constructed a yeast two-hybrid system that employed human estrogen receptor β (hERβ) LBD as a bait and one of p160 family co-activators as a fish (Lee et al., 2002). Although usefulness of this system was demonstrated as a detection system for EDs, we attempted, in this study, further improvement of the system by using full-length hERα that carries both AF-1 and AF-2. We tested various combinations of hERs and co-activators for the efficient ligand-dependent expression of the reporter gene in *S. cerevisiae* and showed that the combination of hERα and the co-activator SRC1 gave a highly sensitive, specific, and reproducible system for detecting estrogenic chemicals.

2. Materials and Methods

2.1. Chemicals

17β-Estradiol (E_2), estrone, and testosterone were purchased from Sigma Chemical Co. Genistein and coumestrol were purchased from Fluka Chemie AG. Bisphenol A (BSA), diethylstilbestrol (DES), 4-*tert*-octylphenol (4-*tert*-OP), and 4-nonylphenol (4-NP) were obtained from Tokyo Kasei Kogyo (Tokyo). γ-Hexachlorocyclohexane (γ-HCH) and 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (*p,p'*-DDT) were obtained from Wako Pure Chemicals (Osaka). 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2,3,5-trichlorophenoxyacetic acid (2,4,5-T) were purchased from Kanto Chemical Co. (Tokyo). All chemicals used were of reagent grade, and used without further purification.

2.2. Plasmids and yeast strains

Construction of pGAL4 DBD-hER??LBD was described previously (Sasagawa et al., 2000; Lee et al., 2002). hERα LBD cDNA (corresponding to amino acid residue 311 to 595 of hERα) was PCR-amplified and inserted between the *Eco*RI and *Sal*I sites of the plasmid

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pGBT9 (Clontech). For construction of pGAL4 DBD-hERα, full-length hERα cDNA was amplified by PCR using a set of primers with either *Eco*RI or *Sal*I site at their 5' termini: hERα Forward/*Eco*RI, 5'-GAATTCATGAC CATGACCCTCCACACC-3'; hERα Reverse/SalI, 5'-GTCGACGCCAGGGAGCTCTCAGAC-3'. The resultant PCR products were once inserted between the *Eco*RI and *Sal*I sites of pBluescript II KS+ (Stratagene) and their nucleotide sequences were determined with a Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer) and an automated DNA sequencer (ABI PRISM 310). Finally, an hERα cDNA fragment of correct sequence was excised form pBluescript II KS+ and inserted between *Eco*RI and *Sal*I sites of pGBT9 to obtain pGAL4 DBD-hERα. Fusion plasmids carrying 'fish' co-activators, pGAL4 TAD-SRC1 NRBD and pGAL4 TAD-TIF2 NRBD, were constructed by inserting co-activator NRBD cDNAs (corresponding to amino acid residue 231 to 1094 of SRC1 and 670 to 1750 of TIF2, respectively) into an *Eco*RI site of the vector pGAD10 (Clontech) (Takeyama et al., 1999; Lee et al., 2002). *S. cerevisiae* YRG-2 (*MAT*α *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 GAL80-538, LYS*::UAS_{GAL1}-$TATA_{GAL1}$-*HIS3*, *URA3*::$UAS_{GAL4\ 17mers\ (X3)}$-$TATA_{CYC1}$-*lacZ*) (Stratagene) was transformed with constructed plasmids (pGAL4 DBD-hERα or pGAL4 DBD-hERα LBD, in combination with pGAL4 TAD-SRC1 NRBD (nuclear receptor binding domain) or pGAL4 TAD-TIF2 NRBD) by the lithium acetate method (Ito et al., 1983). Yeast colonies transformed with the fusion genes were selected on SD medium lacking leucine or tryptophan (Rose et al., 1990). DNA enzymes were used according to the manufacturers' protocols and DNA manipulation was done according to Sambrook et al., 1989.

2.3. Growth of yeast and assay of β-galactosidase activity

β-Galactosidase activity was measured by the method used previously (Lee et al., 2002). Briefly, yeast transformants were grown overnight at 30°C with vigorous shaking in 2 ml selective SD medium. A portion (0.2 ml) of the overnight culture was diluted into 9.7 ml of fresh SD containing test chemicals that were added as dimethylsulfoxide (DMSO) solutions. The amount of DMSO did not exceed 1% of the culture volume. Yeast cells were cultured for 11 h at 30°C. After incubation, cells were collected by centrifugation at 3,500 rpm for 5 min. To prepare crude extracts, Cells were suspended in 1 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol) and disrupted by vortexing together with glass beads for 1 min three times. Cell extract was prepared by removing debris by centrifugation at 15,000 rpm for 10 min. The cell extract (0.2 ml) was mixed with 0.3 ml of fresh Z-buffer and 0.1 ml of 4 mg/ml ONPG (*ortho*-nitrophenyl-β-D-galactopyranoside dissolved in Z-buffer) and incubated at 30°C. After appropriate period, the reaction was terminated by the addition of 0.25 ml of 1 M Na_2CO_3 and absorbance at 420 nm was measured. One unit of enzyme activity was defined as the activity that produced one nmol of *ortho*-nitrophenol per min. The protein concentration of the cell extracts was measured with Bio-Rad Protein Assay (Bio-Rad). The data presented in figures are representative results of three independent experiments, and each experiment was triplicated. Standard error of each data point is indicated with vertical bar.

2.4. Evaluation of estrogenic activities of various compounds using one-hybrid and two-hybrid systems

We evaluated estrogenic activities of the various compounds using one- or two-hybrid systems. The results were converted to REC10 (10% relative effective concentration against E_2) (Nishihara et al., 2000), which is the concentration of a tested chemical showing 10% of the highest estrogenic activity of E_2 in a given system. When the activity of the test substance was higher than REC10 within the concentration tested, we judged the substance as positive (Coldham et al., 1997; Fang et al., 2000).

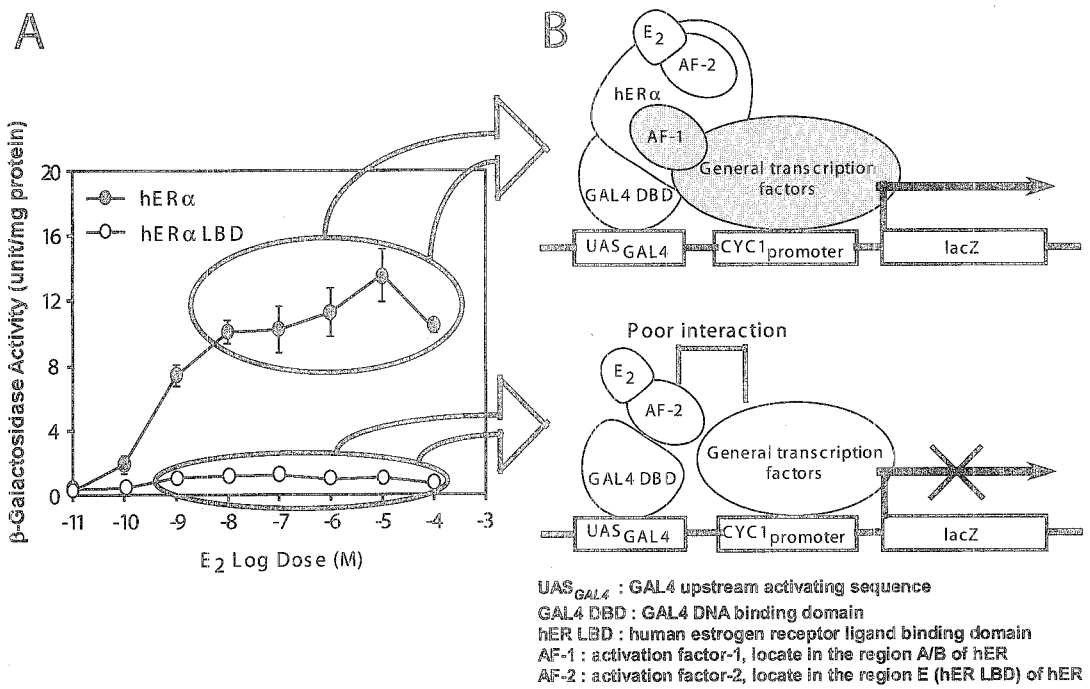

Fig. 1. E_2-Induced β-galactosidase activities by the yeast one-hybrid system. *S. cerevisiae* strain YRG-2 that carried the plasmid pGAL4 DBD-hERα LBD (open circle) or pGAL4 DBD-hERα (closed circle) were incubated in the presence of the indicated concentrations of E_2. β-Galactosidase activities were measured as described in Materials and Methods. Standard error of each point is indicated with vertical bar.

3. Results

3.1. Ligand-dependent activation of the yeast one-hybrid system that employed plasmid pGAL4 DBD-hERα

The gene coding for GAL4 DBD-hERα or Gal4 DBD- hERα LBD fusion protein was expressed from the vector plasmid pGAL4 DBD-hERα or pGAL4 DBD-hERα LBD, respectively, in *S. cerevisiae* strain YRG-2 that had a reporter construct, $UAS_{GAL\ 17mers(X3)}$-$TATA_{CYC1}$-lacZ, on its chromosome. Panel A of Fig. 1 illustrates the response of hERα LBD or hERα to E_2 concentrations of 10^{-11} to 10^{-4} M. Panel B showed outline and principle of the yeast one-hybrid system for estrogenic activity. The YRG-2 strain that expressed GAL4 DBD-hERα showed β-galactosidase activity in response to E_2 of over 10^{-10} M and the highest activity at 10^{-5} M E_2. On the contrary, the same strain that expressed GAL4 DBD- hERα LBD did not show the reporter activity, suggesting that hERα LBD, which carries only AF-2 region, is unable to trans-activate yeast transcription machinery in response to E_2. The entire hERα seems necessary for E_2-dependent trans-activation of the yeast transcription machinery.

We examined the response of this GAL4 DBD- hERα one hybrid system to natural and synthetic steroids and phytoestrogens (Fig. 2A). E_2 showed the weak β-galactosidase activity at 10^{-11} M, but most effective among the chemicals tested at 10^{-5} M. The synthetic estrogen DES effectively induced β-galactosidase activity at more than 10^{-9} M. Estrone, coumestrol, and genistein also induced β-galactosidase activity at more than 10^{-8} M, 10^{-6} M, and 10^{-4} M, respectively. Testosterone did not much effect on this system. Some industrial and pesticide chemicals that had been suspected as EDs also induced β-galactosidase activity (Fig. 2B). Both 4-*tert*-OP and 4-NP induced β-galactosidase activities at over 10^{-5} M, and *p,p'*-

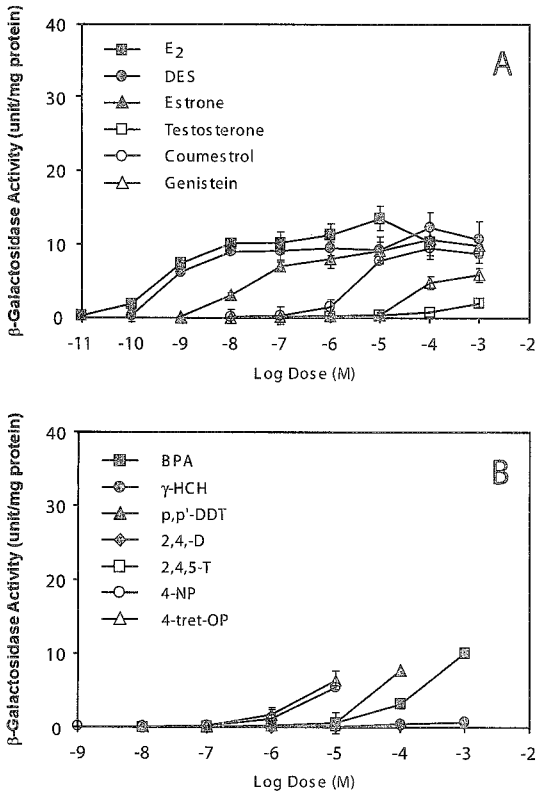

Fig. 2. Dose-response curves for natural and artificial estrogenic chemicals by the yeast one-hybrid system with pGAL4 DBD-hERα. *S. cerevisiae* strain YRG-2 with plasmid pGAL4 DBD-hERα was incubated in the presence of the indicated concentrations of chemicals (panel A show natural and synthetic steroids and phytoestrogens, panel B show industrial and pesticide chemicals). β-Galactosidase activities were measured as described in Materials and Methods. Symbols are designated in figure. Standard error of each point was indicated with vertical bar.

DDT and BPA induced the activity at 10^{-4} M. γ-HCH, 2,4-D, and 2,4,5-T did not induce reporter activity at the tested concentrations. REC10 values of these chemicals in this system are presented in Table 1.

3.2. Ligand-dependent activation of the yeast two-hybrid systems that are based on the interaction between hERα and co-activator NRBDs

The GAL4 DBD-hER and GAL4 TAD-co-activator NRBD fusion proteins were expressed from their expression vectors in *S. cerevisiae* strain YRG-2. In the presence of estrogen, the GAL4 DBD-hER associates with GAL4 TAD-co-activator NRBD and the GAL4 TAD part of the latter protein is expected to efficiently recruit the basal transcriptional machinery to the *CYC1* promoter, resulting in the enhanced production of β-galactosidase. The β-galactosidase activity, therefore, should better reflect the estrogenic potential of tested chemicals (Nishikawa et al., 1999; Sheeler et al., 2000; Lee et al., 2002).

We selected hERα itself or hERα LBD as a fused protein to GAL4 DBD and NRBDs of co-activator SRC-1 or TIF-2 as a fused protein to GAL4 TAD (**Fig. 3**) and examined the response of each combination to E_2. Although the one-hybrid system that expressed only GAL4 DBD- hERα LBD did not respond to E_2, the yeast two-hybrid systems that employed both GAL4 DBD-hERα LBD and GAL4 TAD-co-activator NRBD responded to 10^{-10} M or higher concentrations of E_2 (**Fig. 3**, square symbols). The highest β-galactosidase activity by the system that employed SRC1 NRBD or TIF2 NRBD was 20 units/mg at 10^{-8} M E_2 or 28 units/mg protein at 10^{-6} M E_2, respectively. When GAL4 DBD-hERα was used, combination with GAL4 TAD-SRC1 NRBD or GAL4 TAD-TIF2 NRBD gave much higher β-galactosidase activity at lower E_2 concentrations (**Fig. 3**, circle symbols). The highest β-galactosidase activity by the system that employed SRC1 NRBD or TIF2 NRBD was 26 units/mg protein at 10^{-8} M E_2 or 41 units/mg protein at 10^{-9} M E_2, respectively. These results indicate that, in combination with GAL4 TAD-co-activator NRBD, GAL4 DBD-hERα is more effective in E_2-dependent transcriptional activation than Gal4 DBD-hERα LBD.

3.3. Effect of various estrogenic compounds on the yeast two-hybrid detection system that employed the interaction between hERα LBD and SRC1 NRBD or TIF2 NRBD

In the case of the two-hybrid system in which GAL4 DBD- hERα LBD was combined with GAL4 TAD-

Table 1. Evaluation of estrogenic activities of the various compounds using yeast one-hybrid and two-hybrid systems.

Compound	REC10*				
	One-hybrid system	Two-hybrid system			
	hERα + SRC1	hERα LBD + TIF2	hERα LBD + SRC1	hERα + TIF2	hERα
17β-Estradiol	4.88×10^{-10}	1.75×10^{-10}	2.13×10^{-10}	2.49×10^{-10}	1.22×10^{-10}
Estrone	2.78×10^{-8}	1.60×10^{-8}	1.58×10^{-8}	1.53×10^{-9}	7.90×10^{-10}
Testosterone	3.15×10^{-3}	N	N	1.54×10^{-5}	1.42×10^{-4}
Coumestrol	7.84×10^{-6}	4.56×10^{-6}	2.54×10^{-6}	5.42×10^{-8}	8.92×10^{-7}
Genistein	1.71×10^{-4}	1.33×10^{-4}	1.81×10^{-4}	2.95×10^{-7}	2.35×10^{-6}
DES	1.52×10^{-9}	3.24×10^{-9}	1.45×10^{-9}	3.59×10^{-12}	1.91×10^{-12}
γ-HCH	N	N	nt	1.72×10^{-5}	1.12×10^{-4}
p,p'-DDT	1.25×10^{-4}	nt	1.03×10^{-4}	2.82×10^{-7}	8.35×10^{-7}
2,4-D	N	2.09×10^{-4}	nt	5.42×10^{-6}	6.33×10^{-5}
2,4,5-T	N	N	nt	1.01×10^{-6}	4.35×10^{-6}
BPA	2.05×10^{-4}	1.47×10^{-4}	2.12×10^{-4}	3.93×10^{-6}	1.42×10^{-5}
4-NP	1.07×10^{-5}	5.60×10^{-6}	4.03×10^{-6}	1.37×10^{-7}	1.46×10^{-6}
4-*tert*-OP	5.18×10^{-6}	1.80×10^{-6}	2.41×10^{-6}	1.82×10^{-7}	1.14×10^{-6}

*REC10 (10% relative effective concentration against E_2) is the concentration of a tested chemical showing 10% of the highest estrogenic activity of E_2 in a given system. N, Negative; nt, not tested. β-Galactosidase activities that gave REC10 were 1.4, 2.14, 2.1, 2.8, and 4.1 units/mg protein for hERα one-hybrid system, and for two-hybrid systems of hERα LBD + SRC1 NRBD, hERα LBD + TIF2 NRBD, hERα + SRC1 NRBD, and hERα + TIF2 NRBD, respectively.

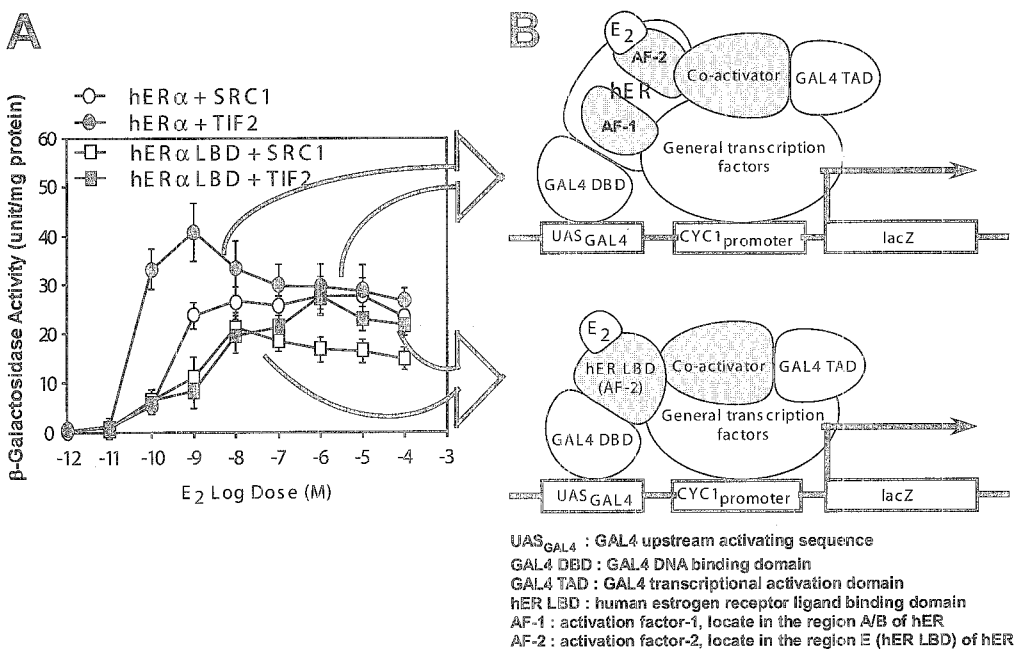

Fig. 3. E_2-Dependent β-galactosidase activities of the yeast two-hybrid systems that express Gal4 TAD-co-activator NRBD. *S. cerevisiae* strain YRG-2 that contained pGAL4 DBD-hERα LBD or pGaAL4 DBD-hERα and pGAL4 TAD-SRC1 NRBD or GAL4 TAD-TIF2 NRBD were incubated in the presence of various concentrations of E_2. Symbols are: open circle, pGAL4 DBD-hERα and pGAL4 TAD-SRC1 NRBD; closed circle, pGAL4 DBD-hERα and pGAL4 TAD-TIF2 NRBD; open triangle, pGAL4 DBD-hERα LBD and pGAL4 TAD-SRC1 NRBD; closed triangle, pGAL4 DBD-hERα LBD and pGAL4TAD-TIF2 NRBD.

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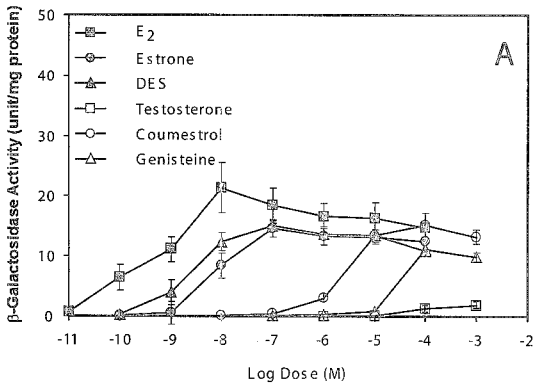


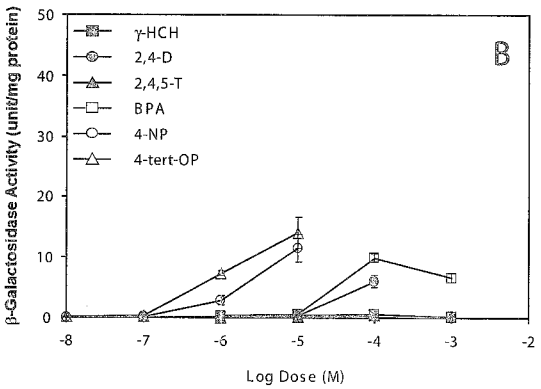
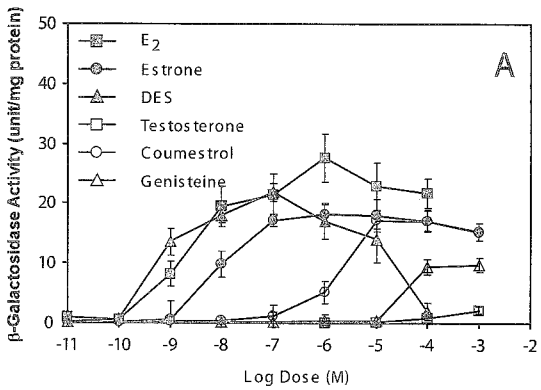


Fig. 4. Dose-response curves for various chemicals as determined by the yeast two-hybrid system with plasmids pGAL4 DBD-hERα LBD and pGAL4 TAD-SRC1 NRBD. Experimental conditions and abbreviations are the same as those in Fig. 2 except for the used plasmids.



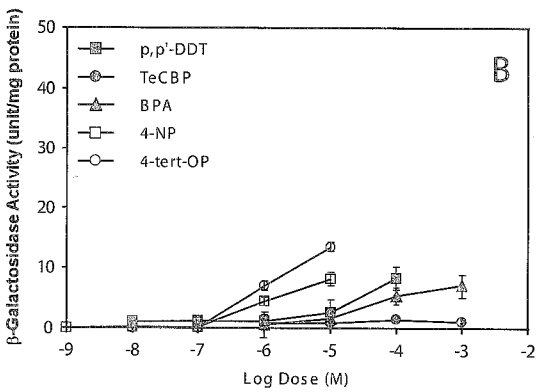


Fig.5. Dose-response curves for various chemicals as determined by the yeast two-hybrid system with plasmids pGAL4 DBD-hERα LBD and pGAL4 TAD-TIF2 NRBD. Experimental conditions and abbreviations are the same as those in Fig. 2 except for the used plasmids.

SRC1 NRBD (Fig. 4), E_2 was the most effective estrogen among the chemicals tested. Estrone and DES were next effective and induced reporter expression at 10^{-9} M or more. Phytoestrogens derived from wood, coumestrol and genistein, were effective at higher concentrations more than 10^{-6} M and 10^{-4} M, respectively. Testosterone was slightly effective only at very high concentrations (Fig. 4A). When pesticides, industrial chemicals and alkylphenols were tested (Fig. 4B), 4-NP and 4-*tert*-OP effectively induced reporter activity at more than 10^{-6} M. 2,4-D and BPA also effective at more than 10^{-4} M, but γ-HCH and 2,4,5-T had little inducible effect on this system. REC10 values of these chemicals are listed in Table 1.

In the case of the two-hybrid system in which GAL4 DBD- hERα LBD was combined with GAL4 TAD-TIF2 NRBD (Fig. 5), E_2 was less effective but induced the reporter activity at 10^{-9} M or more. DES was very effective and induced the reporter activity at more than 10^{-9} M. Inductions by estrone, coumestrol, genistein, and testosterone were very similar to those observed in the above SRC1 NRBD-employed system. When pesticides, industrial chemicals and alkylphenols were tested (Fig. 5B), 4-NP and 4-*tert*-OP induced reporter activities at more than 10^{-6} M. The *p,p'*-DDT and BPA evidently induced the reporter activity at 10^{-4} M or more. TeCBP (PCB) had little effect on this system. REC10 values of these chemicals in this two-hybrid system are listed in Table 1.

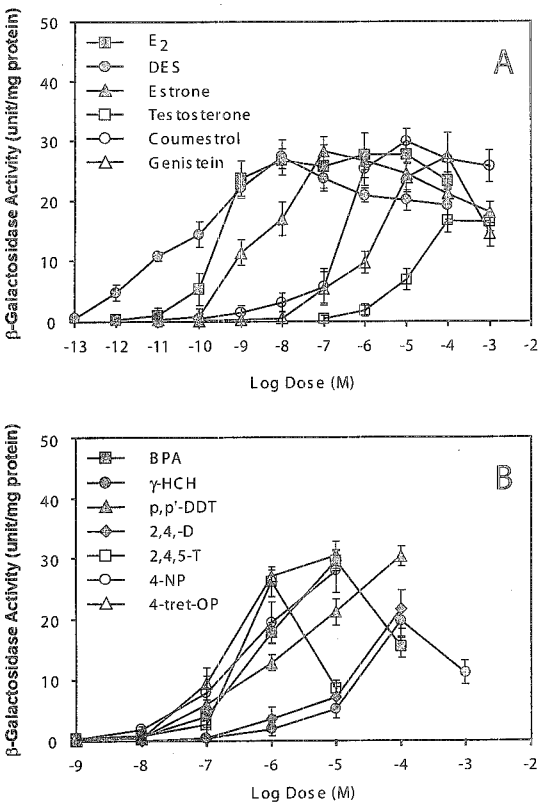


Fig. 6. Dose-response curves for various chemicals as determined by the yeast two-hybrid system with pGAL4 DBD-hERα and pGAL4 TAD-SRC1 NRBD. Experimental conditions and abbreviations are the same as those in Fig. 2 except for the used plasmids.

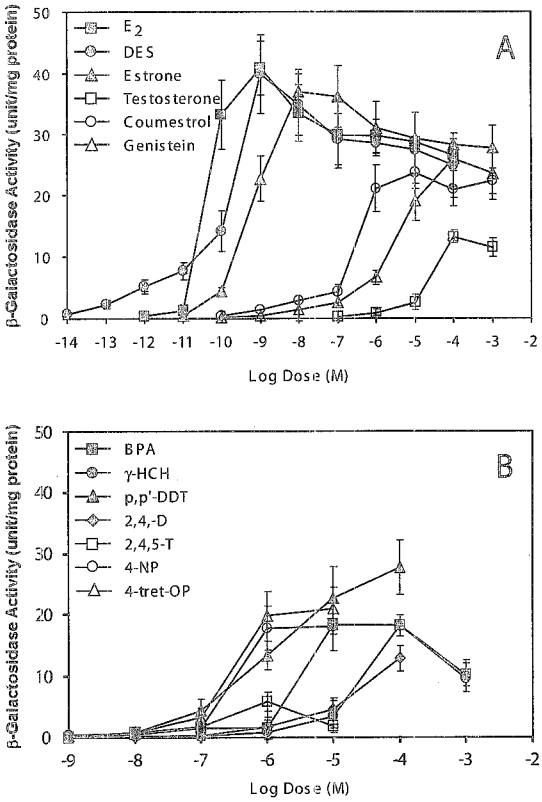


Fig. 7. Dose-response curves for various chemicals as determined by the yeast two-hybrid system with pGAL4 DBD-hERα and pGAL4 TAD-TIF2 NRBD. Experimental conditions and abbreviations are the same as those in Fig. 2 except for the used plasmids.

3.4. Effect of various estrogenic chemicals on the yeast two-hybrid detection system that employed the interaction between hERα and SRC1 NRBD or TIF2 NRBD

The two hybrid systems in which GAL4 DBD- hERα was combined with GAL4 TAD-SRC1 NRBD or GAL4 TAD-TIF2 NRBD were analyzed for their responsiveness to a variety of natural and synthetic steroids and phytoestrogens (Fig. 6A and Fig. 7A). In the case of the combination of GAL4 DBD-hERα and GAL4 TAD-SRC1 NRBD (Fig. 6), E_2 induced β-galactosidase activity at more than 10^{-10} M, whereas DES was further effective and its least induction concentration was 10^{-12} M. Estrone, coumestrol, and genistein were also effective and their least induction concentrations were 10^{-9} M, 10^{-8} M, and 10^{-7} M, respectively. Testosterone induced β-galactosidase activity at 10^{-6} M or more. This system is 10 to 100 times more sensitive to estrogens than the two-hybrid systems that employed hERα LBD and co-activator NRBDs. When pesticides, industrial chemicals and alkylphenols were tested (Fig. 6B), 4-NP induced reporter activity at more than 10^{-8} M. 4-*tert*-OP, *p,p'*-DDT, BPA, and 2,4,5-T also induced reporter activity at more than 10^{-7} M. 2,4-D and γ-HCH are less effective and had similar dosage effect to testosterone at over 10^{-6} M. Their REC10 values in this system are listed in Table 1.

In the case of the combination of GAL4 DBD- hERα with GAL4 TAD-TIF2 NRBD (Fig. 7), E_2 also induced β-galactosidase reporter activity at more than 10^{-10} M.

DES was very effective and its least induction concentration was 10^{-13} M. Estrone, coumestrol, and genistein were also effective and their least induction concentrations were 10^{-10} M, 10^{-8} M, and 10^{-7} M, respectively. Testosterone induced β-galactosidase activity at 10^{-5} M or more. When pesticides, industrial chemicals and alkylphenols were tested (**Fig. 7B**), 4-*tert*-OP and *p,p'*-DDT and induced reporter activity at more than 10^{-7} M. 4-NP induced at more than 10^{-6} M. BPA, 2,4-D and γ-HCH induced reporter activity at 10^{-5} M ore more. 2,4,5-T was slightly effective at only 10^{-6} M. Their REC10 values in this system are listed in **Table 1**.

These two two-hybrid systems that employed GAL4 DBD-hERα in combination with GAL4 TAD-SRC1 NRBD or GAL4 TAD-TIF2 NRBD similarly responded to the estrogens and related compounds. The former combination, however, was more sensitive to pesticides, industrial chemicals and alkylphenols than the latter combination and suitable for the primary screening of a wide variety of potential endocrine-disrupting chemicals.

4. Discussion

In this paper, we presented a useful biosensor as yeast two-hybrid system for the detection of potentially estrogenic chemicals. We first tested yeast one-hybrid systems using hERα and found that the full-length hERα but not hERα LBD was able to trans-activate the yeast transcription machinery in an estrogen-dependent manner. The extent of activation in this system, however, was not high and some chemicals known as estrogenic did not induce reporter activity. We next tested two-hybrid systems that utilized the interaction of hERα or hERα LBD with p160 family co-activator NRBDs and found that the combination of hERα with SRC1 NRBD was most efficient in response to potential estrogenic chemicals.

hERα has two transcriptional activation domains, AF-1 and AF-2. The N-terminus AF-1 was found to be essential for the transactivation in the yeast one-hybrid assay (Bush et al., 1996), where reporter gene expression was dependent on UAS_{GAL}. When hERα with the DNA- and ligand-binding domains and without AF-1 was tested for the estrogen-responsive element (ERE)-dependent transcription induction, it resulted in only weak constitutive expression of reporter activity in yeast (Metzger et al., 1995). These are consistent with our present result in which GAL4 DBD- hERα LBD did not induce reporter activity. The GAL4 DBD-hERα fusion construct gave estrogen-dependent induction of the reporter gene transcription, as expected, and was presumably able to recruit yeast transcription machinery to the reporter gene promoter, $UAS_{GAL4\ 17mers\ (X3)}$-$TATA_{CYC1}$. It has not been fully elucidated how hERα interacts with yeast transcription proteins. The AF-1 alone, in conjunction with the hERα DNA binding domain C or the Gal4 DNA binding domain, was constitutively active in the expression of reporter gene of which promoter contained ERE or UAS_{GAL}, respectively, although the extent of the reporter expression was dependent on its promoter context (Metzger et al., 1992; Metzger et al., 1995). Yeast transcription factors interacting with AF-1 is not yet clear. This activation function of AF-1 is masked in the entire context of hERα and became only functional in the ligand-bound form of hERα. Some yeast transcription factors are known to be involved in ligand-bound hERα-mediated gene activation. Among them, Ada3 and Spt6 interact with the AF-2 region of the ligand-bound hERα (vom Bauer et al., 1998; Benecke et al., 2002). Ada3 also associates with Ada2 and Gcn5, forming the Ada complex that is known to activate the transcription machinery. Interaction of the ligand-bound entire hERα with these transcription factors seems necessary for hERα-induced transcription in yeast.

Co-expression of Gal4 TAD-p160 co-activator NRBD fusion protein much improved ligand-dependent activation of transcription by both GAL4 DBD- hERα LBD and GAL4 DBD-hERα. This is because p160 co-activator NRBD interacts with ligand-bound hERα LBD through NR binding signature motif (LXXLL, NR box). The employed SRC1 and TIF2 NRBDs contained three such motifs, respectively, and one of activation domains,

AD1, which much contributed for transcription activation in yeast (Heery et al., 1997; Sheppard et al., 2003). The use of GAL4 DBD-hERα resulted in higher response to estrogens than those of GAL4 DBD- hERα LBD and GAL4 DBD-hERβ (Lee et al., 2002). This is in part brought about by the synergistic action of AF-1 and AF-2 of hERα, which cooperatively mediates recruitment of co-activator SRC-1 (Metivier et al., 2001), although detailed molecular mechanisms have not been given. A ligand-dependent direct interaction between the B domain in AF-1 and C-terminal domains of ERα was also reported and supposed to stabilize this cooperative interaction between hERα and SRC-1. E_2 is known to be equally effective in binding to the two ER subtypes and generally gives higher transcriptional activation to hERα than to hERβ in cultured cells and yeast two-hybrid systems (Pettersso and Gustafsson, 2001; Lee et al., 2002). It is also reported that the type of NR box in SRC-1 NRBD is responsible for the ligand-dependent binding affinity of NR subtypes to SRC-1 (Bramlett et al., 2001).

To compare the sensitivity of the system here presented with others that have appeared in publications, chemicals and their REC10 concentrations were listed in **Table 1**. All chemicals tested by the two-hybrid systems that employed GAL4 DBD-hERα and GAL4 TAD-SRC1 NRBD or GAL4 TAD-TIF2 NRBD were positively estrogenic. On the contrary, γ-HCH, 2,4-D, and 2,4,5-T were negative by the GAL4 DBD-hERα one-hybrid assay. Testosterone, γ-HCH, and 2,4,5-T were negative by the two-hybrid assay that employed GAL4 DBD- hERα LBD and GAL4 TAD-SRC1 NRBD. Testosterone and TeCBP were negative by the two-hybrid assay that employed GAL4 DBD- hERα LBD and GAL4 TAD-TIF2 NRBD. These findings are in good agreement with those in previous reports, which demonstrated estrogenic activities of suspected chemicals by *in vivo* and *in vitro* assays, with the exception of some chemicals that we tested first in this report (Soto et al., 1995; Olea et al., 1996).

Recently, Ellison et al. reported a yeast detection system for endocrine disruptors, in which ERα and SRC-1 interaction was utilized (Ellison et al., 2003). hERα and SRC-1a, a subtype of SRC-1, were expressed in yeast that carried a reporter gene *lacZ*, promoter of which had three estrogen-responsive elements. They found the estrogen and SRC-1 dependent reporter gene expression, although the level of the reporter activity was two to three orders lower than those by our system. This is probably because of the inefficiency of trans-activation by SRC-1 itself in yeast. This biosensor is clear that the present two-hybrid system that utilizes the interaction between the entire hERα and the p160 co-activator on the basis of Gal4 DNA binding and trans-activation functions could be very useful and enable the detection of estrogenic activities of natural hormones and endocrine disruptors at one to three orders lower concentrations than the previous yeast systems.

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