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Basis of a High-Throughput Method for Nuclear Receptor Ligands

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Assessment of the risk of human exposure to man-made chemicals that bind to hormone receptors has emerged as a major public health issue. Among hormone receptors, nuclear receptors tend to be targets of xenobiotics because their endogenous ligands are small, fat-soluble molecules. Nuclear receptors are ligand-inducible transcriptional factors and regulate the transcriptional activity of various target genes. At the start of the initiation step of transcription, nuclear receptors interact with coactivators (TIF2, SRC1, ACTR, CBP/p300, *etc.*) in an agonist-dependent manner. Using the interaction of the nuclear receptor with a coactivator, we have developed a novel rapid ligand *in vitro* screening method that is easy to use and has high sensitivity. This method, called by us the CoA-BAP system, is applicable to most nuclear receptors and is suitable for high-throughput screening because the entire experimental operation can be carried out on a microplate. We used human TIF2 as a coactivator including LXXLL motifs expressed in *Escherichia coli* as a fusion protein with BAP and nuclear receptor LBD expressed in *E. coli* as a fusion protein with GST. On a GSH-coupled microplate these proteins were incubated with chemicals and the protein-protein interactions were detected as alkaline phosphatase activity. To date we have examined seven nuclear receptors (ERα/β, TRα, RARα/γ, RXRα,and VDR) and confirmed that the method works well.

Key words: endocrine disruptor, *in vitro* screening method, hormone receptor, human nuclear receptor, TIF2.

Abbreviations: AP, alkaline phosphatase; ER, estrogen receptor; GST, glutathione-S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; LBD, ligand binding domain; NR, nuclear receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; TIF2, transcriptional intermediary factor 2; TR, thyroid hormone receptor; VDR, vitamin D receptor.

Nuclear receptors play important roles in the maintenance of the endocrine system, regulation of organ differentiation and fetal development. Recently, the human genome was reported to contain 48 members of the nuclear receptor family. This superfamily includes not only the classic endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, fat-soluble vitamins and fatty acids, but also a large number of so-called orphan receptors, whose ligands were initially unknown (*1*).

Disruption of the hormone system, especially sex steroid hormones, has adverse effects on reproduction, organ differentiation and fetal development. Therefore, the physiological functions of what have attracted considerable attention. However, their functional details remain to be elucidated. In particular, the discovery of estrogenic compounds (*2*) has received much attention, and this has facilitated explanation the mechanism of so-called endocrine disruptors.

Nuclear receptors commonly possess particular structures in several regions (A/B, C, D, and E/F). The A/B region contains the AF-1 region, which mainly regulates ligand-independent transcriptional activity. On the other hand, the E/F region contains the AF-2 region, which regulates ligand-dependent transcription through ligand binding and dimerization as homo- or hetero-dimers with RXR (*3*, *4*). Also, this region interacts with several coactivator proteins including CBP/p300 (*5*) and p160 family members, such as SRC1 (*6*), TIF2 (*7*), ACTR (*8*), and so on. The C region is the most conserved region, and contains two zinc-finger modules responsible for DNA-binding and sequence specific recognition (*9*).

Regarding transcriptional initiation triggered by a ligand, the interaction of the E/F region with a coactivator is very important. Many factors have been identified as nuclear receptor coactivators; these include CBP/p300, p160 family members (*10*), TRAP220 (*11*), and TIP60 (*12*). All of these coactivators contain several LXXLL motifs capable of interacting with nuclear receptors (*13*, *14*). This motif is known to create the hydrophobic surface that is able to interact with the ligand binding pocket composed of α-helices H3, H5/6, and H12 in the E/F region of nuclear receptors (*15–20*). In contrast, several proteins called co-repressors (SMRT and NCoR) have several LXX I/H IXXX I/L motifs that create a similar but different hydrophobic surface. The ligand binding pocket of an apo-receptor or antagonist-bound receptor has its lid put on by the LXX I/H IXXX I/L motif (*21*, *22*).

Among coactivators, p160 family members have often been used as models for study ligand-inducible interactions with nuclear receptors. Moreover, p160 coactivators are known to be capable of interacting with most nuclear

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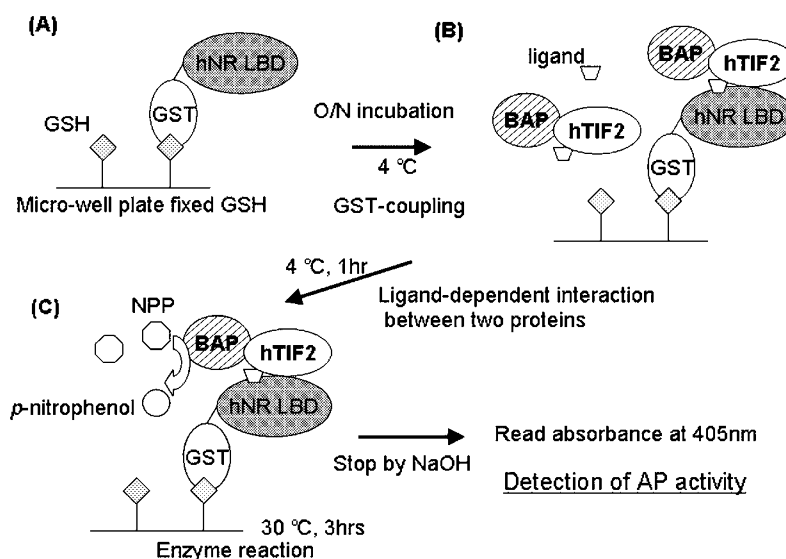

Fig. 1. **Scheme of a new screening method for human nuclear receptor ligands.** A flowchart of the CoA-BAP system is presented. (A) A crude *E. coli* extract including the GST-hNR LBD protein was incubated on micro-well plate fixed glutathione (GSH). (B) After overnight incubation at 4°C, excessive protein was removed. Then, the hTIF2 NID-BAP fusion protein was added with the test chemical. (C) After one hour incubation at 4°C, excessive protein was removed carefully. NPP (*p*-nitrophenylphosphoric acid) was added as a substrate for BAP, and the enzyme reaction was started at 30°C. After 3 h, the reaction was stopped by the addition of 0.5 N NaOH. The yellow color was measured by reading the absorbance at 405 nm with a plate-reader. By subtracting the background value from the value, AP activity was determined.

receptors. In our previous report, we proposed a rapid screening method for chemicals with hormonal activities involoving the interaction between the coactivator NID (nuclear receptor interaction domain) and the LBD (ligand binding domain) of several nuclear receptors in yeast (*23*). This system, the yeast two-hybrid assay, is a very simple and inexpensive test method with good repeatability. However, the permeability of xenobiotics through the cell membrane and toxicity to the yeast itself have been pointed out to be problems that should be taken into consideration.

In this paper, we report the development of an *in vitro* detection system for protein-protein interactions on a microplate involving a GST-NR (nuclear receptor) fusion protein and a BAP-CoA (coactivator) fusion protein. This method, designated as the CoA-BAP system (Fig. 1), could be adapted to a wide range of nuclear receptors. Although there are several assay methods for the evaluation of nuclear receptor ligands (*24*), they are often complicated to perform or costly. Our new method is simple, inexpensive and not time-consuming. Moreover, it can be adapted to high-throughput screening because all steps are performed on a single plate.

MATERIALS AND METHODS

Plasmids—The NID (nuclear receptor interaction domain) of human TIF2 cDNA was amplified by RT-PCR using human liver mRNA as a template. The primer sets used for amplification synthesized were as follows: f-primer, 5′-CCCCACTCAGCAAGATGGGA-3′, r-primer, 5′-GCTGTGGTAATTGACTATTC-3′. The entire sequence of the amplified fragment was confirmed by sequencing after subcloning into pBluescript. The fusion genes of hTIF2 NID (codons 573–820) and BAP (bacterial alkaline phosphatase) were subcloned into the *Bam*HI–*Kpn*I sites of pET28a-SKPH that comprised pET28a (Novagen, Madison, WI) with *Kpn*I–*Pst*I sites inserted between its *Sal*I–*Hind*III sites. The LBDs of human ERα (codons 247–595) and ERβ (213–530), TRα (119–410), RARα (170–462), RARα (172–454), RXRα (201–462), and VDR (91–427) were amplified by RT-PCR using human tissue mRNA from ovary, liver, testis and kidney. The LBDs of human nuclear receptors were subcloned into pGEX-4T (Amersham Bioscience, Uppsala, Sweden). All sequences synthesized by PCR were confirmed by DNA sequencing using a DSQ-1000S DNA sequencer (Shimadzu, Kyoto).

Chemicals—17β-Estradiol (E2; >97%), *o-t*-butylphenol (98%), benzyl butyl phthalate (98%), and benzophenone (98%) were purchased from Wako Pure Chemical Industries (Osaka). 3,3′,5-Triiodo-L-thyronine (T3; >97%), all-trans-retinoic acid (ATRA; 98%), 9-*cis* retinoic acid (9-cisRA; 98%), and 1α-25-dihydroxycholecalciferol (Vitamin D3; >99%), were purchased from Sigma-Aldrich Co. (St. Louis, MO). *p-n*-Nonylphenol and *p-n*-butylphenol were purchased from Kanto Chemical (Tokyo). Molecular biological reagents, unless otherwise stated, were from Toyobo (Osaka). All other chemicals were of reagent grade, obtained from commercial sources, and used without further purification.

Preparation of Proteins—The histidine-tagged hTIF2 NID-BAP fusion protein was expressed in the *Escherichia coli* BL21 (DE3) pLysS strain (Stratagene, CA, USA) and purified on Ni-nitrilotriacetic acid (NTA) agarose resin (QIAGEN GmbH, Hilden, German). The GST-hNR LBD fusion proteins, except hERβ, were expressed in the *E. coli* BL21 (DE3) pLysS strain according to the standard procedure (Amersham Bioscience, Uppsala, Sweden). In the case of hERβ, the cells were grown to an optical density = 0.4–0.6 and then grown for a further six hours in the presence of 0.1mM IPTG at 16°C. Bacteria were disrupted by sonication in suspension buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 100 mM KCl, 10% glycerol) supplemented with a proteinase inhibitor cocktail (Nacalai Tesque, Kyoto). The supernatant obtained on centrifugation was used for fixation on the microplate.

GST-Coupling on a Microplate—Glutathione Immobilizer™ microplate (EXIQON A/S, Vedbaek, Denmark), comprising glutathione coupled to a micro-well plate, was used to fix the GST-NR fusion protein on the micro-wells. The micro-well plate was incubated with crude *E. coli* extracts at 4°C overnight in a total volume of 100 μl.

Alkaline Phosphatase Activity—In principle, all experiments were carried out on ice, unless otherwise stated. A

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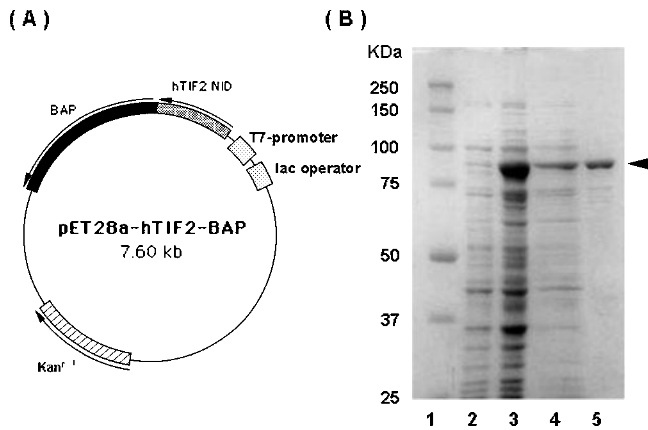

Fig. 2. **Purification of the his-tagged hTIF2 NID-BAP fusion protein.** (A) The his-tagged hTIF2 NID-BAP fusion protein expression vector is depicted. His-tagged hTIF2-NID including three LXXLL motifs fused to BAP at the C-terminal of the protein. (B) 8.5% SDS-PAGE analysis of the purified protein involving Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) affinity chromatography. The fusion protein was efficiently induced on the addition of 1 mM IPTG and purified (arrowhead). Lane 1, size markers; lane 2, uninduced *E. coli* BL21 whole cells; lane 3, 1 mM IPTG induced whole cells; lane 4, supernatant obtained on centrifugation; lane 5, purified his-hTIF2 NID-BAP.

prepared micro-well plate was rinsed with 120 μl of suspension buffer, followed by the further two rinses with 150 μl of suspension buffer. Next, 100 μl of purified histidine-tagged hTIF2 NID-BAP protein (30 μg/ml) was added to the plate with an appropriate concentration of a chemical or control solvent. After 1 h incubation at 4°C, the plate was washed with 120 μl of wash buffer (50 mM Tris-HCl [pH 7.2], 5 mM $MgCl_2$, 100 mM KCl, 0.1% Nonidet P-40). Two further washes were then carried out with 150 μl of wash buffer. After removal of wash buffer, the enzyme reaction was started by the addition of 100 μl of a 10 mM NPP solution (100 mM Tris-HCl [pH 8.0], 10 M *p*-nitrophenylphosphate [Nacalai Tesque, Kyoto]). After incubation at 30°C for 3 h, the reaction was stopped by the addition of 25 μl of 0.5 N NaOH. Finally, the absorbance at 405 nm was measured with a MultiskanJX (Thermo Bio Analysis, Japan). Alkaline phosphatase (AP) activity without GST-NR was also measured as a background for this assay.

RESULTS

Purification of the His-Tagged hTIF2-BAP Fusion Protein—At the beginning, we determined the minimum region of TIF2 that is sufficient for stable binding to ERα. Because the molecular weight of the BAP protein is 52 KDa, it is necessary to minimize the TIF2 portion in order to achieve a high level of expression in *E. coli* as a soluble protein. The region (a.a. 573–820) of TIF2 containing three LXXLL motifs was determined to be best for our purpose. A 75 kDa fusion protein, which was consistent with the expected size, was efficiently expressed and purified by Ni-agarose affinity chromatography (Fig. 2).

Expression of the GST-Human NR LBD Fusion Protein—Ten percent SDS–polyacrylamide gel electrophoresis analysis of various nuclear receptor LBDs is shown in Fig. 3. Purification from a crude *E. coli* extract with Glutathione Sepharose 4B (Amersham Bioscience, Uppsala, Sweden) indicated that these GST-nuclear receptor (GST-NR) fusion proteins were induced efficiently on the addition of IPTG, and mainly existed in the soluble fraction.

Ligand-Dependent Interaction between GST-hNR and hTIF2-BAP—Using the above recombinant proteins, we examined the ligand-dependent interaction between GST-hNRs and hTIF2-NID. In the hERα/β system, 17β-estradiol enhanced the AP activity from 100 pM (Fig. 4A). In the hRARα/γ system, all-trans retinoic acid enhanced the AP activity from 1 nM (Fig. 4, B and C). In the hTRα and hVDR systems, each ligand, 3,3′,5-triiodo-L-thyronine (T3) and 1α-25-dihydroxycholecalciferol (vitamin D3), enhanced the AP activity from 10 nM (Fig. 4D). In the hRXRα system, 9-*cis* retinoic acid also enhanced the AP activity from 100 nM (Fig. 4C).

All systems gave good dose-response curves, which proved that this new method works well and is useful. We

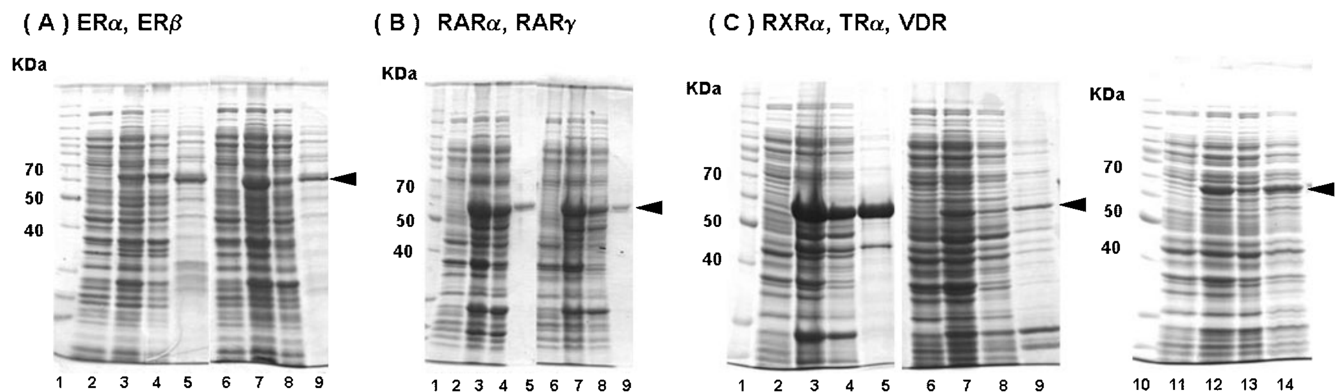

Fig. 3. **GST-hNR protein patterns in *E. coli* crude extracts. 10% SDS-PAGE analysis of crude *E. coli* extracts including GST-fusion hNR LBD (ligand binding domain) proteins is shown: (A) hERα/β, (B) hRARα/γ, (C) hRXRα, hTRα, hVDR, respectively. Their hNR LBDs were purified by Glutathione Sepharose 4B (Amersham Bioscience) affinity chromatography.** The efficient purification of the GST-hNRs suggested the GST-hNR LBD proteins were good enough to be fixed on a GSH-fixed microplate. (A) Lanes 2–5, hERα; lanes 6–9; hERβ (B) lanes 2–5, hRARα; lanes 6–9, hRARγ (C) lanes 2–5, hRXRα; lanes 6–9, hTRα; lanes 11–14, hVDR. Lanes 1, 5, 10, size markers; lanes 2, 6, 11, uninduced *E. coli* BL21 whole cells; lanes 3, 7, 12, IPTG-induced whole cells; lanes 4, 8, 13, supernatants obtained on centrifugation; lanes 5, 9, 14, purified GST-hNR (arrowhead).

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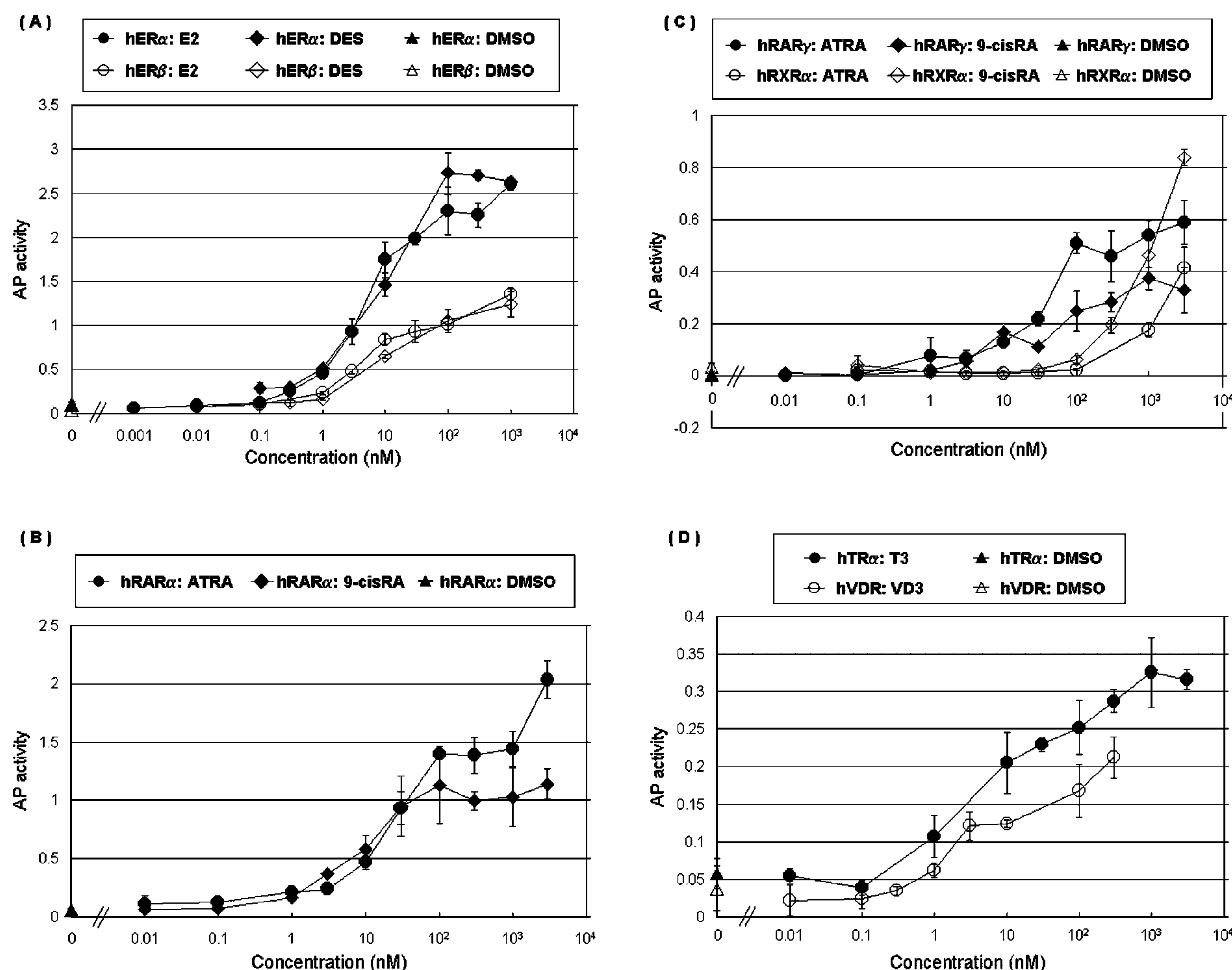

Fig. 4. **Dose-response curves of endogenous ligands for various nuclear receptors.** Lignd-dependent interactions between GST-hNR and hTIF2-BAP were determined as AP activity (as described under Methods). AP activity without GST-NR was measured as a background for this assay. Means ± SD (n = 3) are shown. 17β-Estradiol was used as an endogenous ligand for hERα/β, and DES as a known powerful ligand (A). ATRA was used as an endogenous ligand for hRARα (B) and hRARγ (C), and as weak ligand for hRXRα (C). 9-cisRA was used as a powerful ligand for hRXRα and hRARs. T3 was used as an endogenous ligand for hTRα, and vitamin D3 as an endogenous ligand for hVDR (D).

suggest that our system is as good as TR-FRET (Time resolved Fluorescence Resonance Energy Transfer) analysis, one of the high-sensitivity methods for the detection of interactions between nuclear receptors and coactivators (*25–28*).

When we carried out a control experiment with GST alone (without a nuclear receptor), little activity was detected, *i.e.* the same level as the background AP activity (data not shown). So, we can say that the detected AP activity was derived from interaction between nuclear receptor LBD and hTIF2-NID.

Application of CoA-BAP System to Xenobiotics—We next examined application of the CoA-BAP system to evaluation of the hormonal activity of xenobiotics (Fig. 5 and Table 1). We tested some suspected endocrine disruptors; *p-n*-nonylphenol, *p-n*-butylphenol, *o-t*-butylphenol, benzophenone; benzene derivatives, benzylbutyl phthalate (BBP); phthalate, and dietylstilbesterol (DES). With the CoA-BAP system, we detected strong activity for DES and moderate activity for *p-n*-butylphenol with the hERα/β-hTIF2 system. BBP caused weak enhancement of the AP activity in the hERα-hTIF2 system (Fig. 5). The patterns of responses to chemicals were consistent with the estrogenic activity measured with other assay systems (*23*, *29*).

With other receptors, *o-t*-butylphenol exhibited weak activity for hTRα, hRARs and hRXRα, but no activity for ERs. All tested alkylphenols except *p-n*-nonylphenol exhibited very weak activity for hTRα, hRARs, and hRXRα at 100 μM (Table 1). These results suggested that alkylphenols might exert endocrine disruptive effects mediated by multiple nuclear receptors.

DISCUSSION

Previously we proposed a screening method for chemicals with hormonal activities involving a yeast two-hybrid assay (*23*). We examined about five hundred chemicals

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Table 1. **Dose dependency of chemicals for the hNR-hTIF2 interaction in the CoA-BAP system.**

Receptor	hERα	hERβ	hTRα	hVDR	hRARα	hRARγ	hRXRα
Endogenous ligand/lowest effective conc.	17β-Estradiol (E2)/100 pM	17β-Estradiol (E2)/100 pM	3,3′,5-Triiodo-L-thyronine (T3)/10 nM	1α-25-Dihydroxy cholecalciferol (VD3)/10 nM	all-*trans* Retinoic acid (ATRA)/1 nM	all-trans Retinoic acid (ATRA)/1 nM	9-*cis* Retinoic acid (9-cisRA)/ 100 nM
Tested chemical							
p-*n*-Nonylphenol (*p*-*n*-NP)	–	–	–	–	–	–	–
p-*n*-Butylphenol (*p*-*n*-BP)	+	+	–	–	+	+	+
o-*t*-Butylphenol (*o*-*t*-BP)	–	–	+	–	+	+	+
Diethylstilbestrol (DES)	+++	+++	–	–	–	–	–
Benzyl butyl phthalate (BBP)	+	–	–	–	–	–	–
Benzophenone (BZP)	–	–	–	–	–	–	–

+++: Strong activity was observed, the same as the endogenous ligand level. +: Weak activity was observed, one-hundred thousand times lower than the endogenous ligand level. –: No activity was observed with the tested concentration.

including suspected endocrine disruptors in the rat ERα-hTIF2 system (*29*). In past examinations we faced some problems, *i.e.* the permeability of the cell membrane to xenobiotics and toxicity to the yeast cells. In addition, we needed a more rapid method to enable us to examine the effects of numerous chemicals on the many kinds of nuclear receptors.

Therefore, as reported here, we have developed a new screening method for nuclear receptor ligands that overcomes these problems of previous assay systems. There are several *in vitro* methods for detecting ligands for nuclear receptors; reporter gene assays, pull-down assays, competitive binding assays, and TR-FRET analysis. The yeast two-hybrid assay showed improved repeatability and reduced cost. However, the sensitivity of the yeast two-hybrid assay was inferior to these of previous methods by a concentration factor of about one order of magnitude. Although TR-FRET analysis (*25–28*) is superior to other methods including the reporter gene assay, it needs expensive instrumentation such as a time-resolved fluorescence reader.

We have developed a method involving the GST-NR fusion protein expressed in *E. coli*, and a glutathione-coupled microplate to which nuclear receptors can be easily fixed. Using the TIF2-BAP fusion protein as a detection reagent, our method became easier, faster and less expensive. In addition, this method (CoA-BAP system) is suitable for high-throughput screening because all steps can be conducted on a microplate. The utilization of fusion proteins comprising a binding domain and a reporter domain has the advantage of easy detection of specific binding events (*30*).

We tested several nuclear receptors with the CoA-BAP system, such as hERα/β, hTRα, hRXRα, hRARα/γ, and hVDR. For all nuclear receptors tested we could detect an increase in alkaline phosphatase activity in a ligand-dependent manner. The sensitivities for 17β-estradiol were shown from 100 pM with both hERα and β systems. With other receptor systems the sensitivities for each endogenous ligand were from 1 nM to 100 nM. These results suggest that this new method is better than our previous method, *i.e.* the yeast two-hybrid assay. While the CoA-BAP system has many benefits, it requires soluble proteins. However, some fusion proteins of GST with nuclear receptors (*e.g.* AR and GR) tend to aggregate in *E. coli.* In such cases, the baculovirus expression system might be beneficial.

Next, we applied our new method to xenobiotics, including some suspected endocrine disruptors. With the exception of *o*-*t*-butylphenol, we previously reported the results for these compounds as to estrogenic activity with the rat ERα system obtained using the yeast two-hybrid

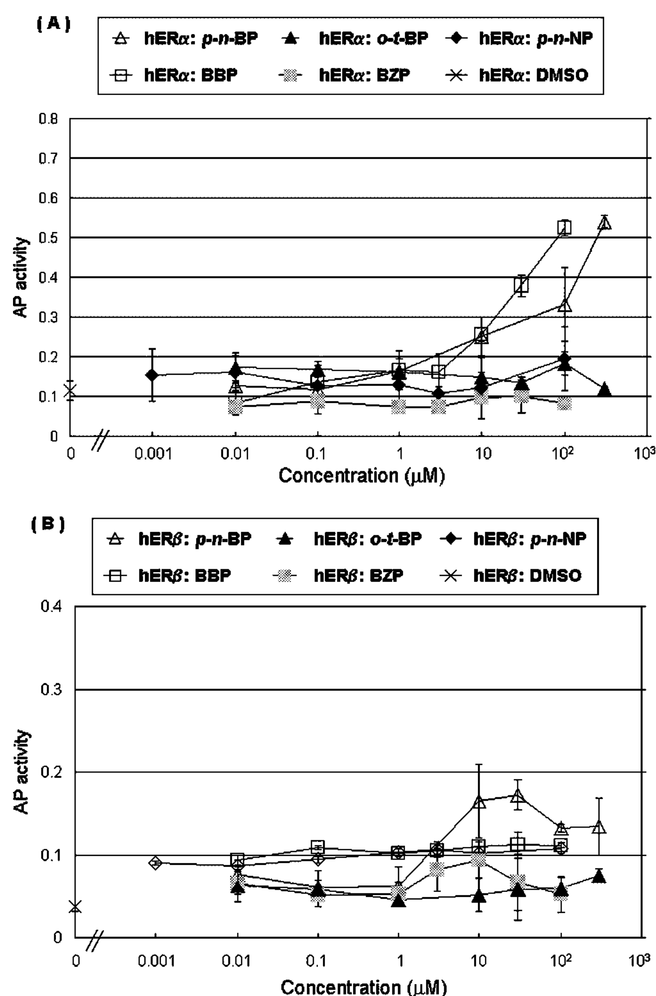

Fig. 5. **Application of the CoA-BAP system to xenobiotics.** Some suspected endocrine disruptors were examined for hormonal activity. The results with hERα/β systems are shown. The results with other receptors are shown together in Table 1. AP activity without GST-NR was measured as a background for this assay. Means ± SD (n = 3) are shown.

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assay. As expected, we detected strong activity for DES and moderate activity for *p-n*-butylphenol. Taking into consideration our previous data, we consider that our new method was efficiently applicable to xenobiotics. In addition, we found that *o-t*-butylphenol exhibited slight activity for hTRα and hRARs. *p-n*-Butylphenol showed slight activity with hRARs as well as ER. These results suggested that weak hormonal activities of xenobiotics can be detected by the CoA-BAP system.

Recently, the rapid development and application of molecular biological techniques have revealed the whole human genome. From the result of analysis of genome sequences we know that the human nuclear receptor superfamily consists of 48 members. However, 25 receptors have been recognized as orphan nuclear receptors, whose ligands have not so far been identified (*1*, *4*, *31*). By analogy with known nuclear receptor ligands, these orphan nuclear receptors might have important physiologically active ligands. For this reason orphan nuclear receptors have attracted much attention as targets for novel drug discovery (*32–37*). By virtue of its comprehensive applicability and speed, the CoA-BAP system will become a powerful tool as a screening method for ligands of orphan nuclear receptors.

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