Liquid chemiluminescent DNA pull-down assay to measure nuclear receptor-DNA binding in solution

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Electrophoretic mobility shift assays (EMSAs) are commonly used to investigate protein-DNA binding in vitro. However, EMSA can generate considerable amounts of undesirable waste, particularly when toxic compounds are examined. We therefore developed a novel in vitro protein-DNA binding assay called liquid chemiluminescent DNA pull-down assay, which is based on solution hybridization between digoxigenin-labeled DNA and glutathione S-transferase (GST)-fused DNA binding protein bound to glutathione-Sepharose beads.

INTRODUCTION

Protein-DNA interaction in vitro is usually detected by electrophoretic mobility shift assays (EMSAs) (1,2) that require radioisotopically labeled DNA and autoradiographic visualization (Figure 1A). These lengthy processes generate radioactive and toxic waste. Furthermore, detection of weak binding is difficult because protein-DNA complexes must migrate through polyacrylamide gels. A nonradioisotope-based EMSA system has been developed to avoid radioactive contamination (3). However, gel electrophoresis and transfer of separated DNA-protein complex to a membrane are required, which also generates waste. The disposal of large volumes of toxic and/or radioactive waste can be problematic, particularly when toxic compounds are examined.

We recently demonstrated that several polychlorinated biphenyl (PCB) congeners known to be endocrine disrupting chemicals suppress transcription through thyroid hormone receptor (TR), a ligand-regulated transcription factor, by dissociating TR from thyroid hormone response element (TRE) (4,5). Because thyroid hormone plays a critical role in brain development and because perinatal exposure to PCB and/or dioxin may produce abnormal brain development similar to that seen in hypothyroid mammals (6,7), we started

screening a series of known PCB and dioxin congeners with chemical structures similar to that of PCB (8) for their ability to disrupt TR binding to TRE. PCB has 209 congeners, whereas dioxin has 210 (8). Furthermore, various other chemicals also have similar structures to PCB/dioxins (9). Since EMSA might not be an ideal or appropriate technique for such massive screening for the reasons stated above, we developed a novel liquid chemiluminescent DNA pull-down assay that uses a digoxigenin (DIG)-labeled DNA with a nuclear hormone response element (NRE) and glutathione *S*-transferase (GST)-nuclear receptor (NR) fusion protein (Figure 1B, 1). The protein complex and DNA are hybridized in solution and are detected without electrophoresis by a chemiluminescent reaction. This procedure is much more rapid than EMSA, and it generates far less waste.

Double-stranded oligonucleotides (100 ng) containing an NRE are labeled with DIG using DIG Gel Shift Kit (Roche Diagnostics, Basel, Switzerland) to a final concentration of 4 ng/ μ L of the labeled oligonucleotide. Liquid chemiluminescent DNA pull-down assays can be performed using either DIG-labeled or biotinylated DNA. We purify the GST-fusion protein using glutathione-Sepharose 4B (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instruction. In brief, GST-fusion

protein is produced from a GST expression vector in *Escherichia coli* (5). The 1 mL glutathione-Sepharose 4B is incubated with 3–4 mL sonicated bacteria containing the GST-fusion protein in 30–40 mL GST binding buffer [75 mM KCl, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 0.1% Triton X-100] for 4–16 h at 4°C. During this step, GST-fusion protein is bound to glutathione-Sepharose beads. After washing the beads with GST binding buffer, the GST-fusion protein bound to glutathione-Sepharose beads are stored in GST binding buffer at 4°C.

GST-fused NR (~2 μ g/reaction) bound to glutathione-Sepharose 4B beads is incubated with 10–20 fmol DIG-labeled double-stranded DNA fragments in protein-DNA binding buffer from the kit [20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 0.2% (w/v) Tween 20, 30 mM KCl] in a final volume of 20 μ L for 1 h at room temperature to bind GST-fused NR on beads to DIG-labeled DNA. GST-fused proteins bound to DNA on beads are washed three times with ~1 mL washing buffer from the kit [0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween 20] with or without ligand, then the beads are incubated in ~500 μ L blocking solution [10% (w/v) 10 $\times$ blocking solution in maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl; adjust with NaOH to pH 7.5] from the kit for 30 min before incubation with anti-DIG antibody Fab-fragments conjugated with 75 mU/mL alkaline phosphatase for 30 min. Beads are washed twice with washing buffer from the kit before incubation in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) from the kit for 2–5 min. Beads are transferred to 96-well plates and incubated with 1 μ g/mL chemiluminescent substrate disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) working solution for 5 min at room temperature followed by 10 min at 37°C to enhance CSPD chemiluminescent reaction. Light emission was measured using a MicroLumatPlus LB 96V luminometer (Berthold Technologies, Bad Wildbad, Germany). Our procedure produces results in about one-tenth of the time, while generating about 10% of the waste associated with EMSA. Using this

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Figure 1. Assay methods to detect DNA-protein binding. (A) Representation of electrophoretic mobility shift assay (EMSA). Radioisotope (RI)-labeled DNA and in vitro-translated nuclear receptor (NR) are incubated, and then the mixture is resolved by electrophoresis on polyacrylamide gels, dried, and visualized by autoradiography. (B) Representation of liquid chemiluminescent DNA pull-down assay. 1) Detection of NR-DNA binding. A glutathione *S*-transferase (GST)-fused NR (GST-NR) bound to glutathione-Sepharose beads is incubated with a digoxigenin (DIG)-labeled double-stranded DNA fragment containing a nuclear hormone response element (NRE) in protein-DNA binding buffer. After extensive washing, protein-DNA binding on beads is detected using anti-DIG antibody conjugated to alkaline phosphatase, which is measured by a chemiluminescent reaction using a luminometer. 2) Detection of cofactor-NR-DNA binding. A GST-fused cofactor bound to glutathione-Sepharose beads is incubated with in vitro-translated NR in protein-protein binding buffer for 1 h at room temperature or for 16 h at 4°C. After washing, the DIG-labeled double-stranded DNA fragment containing NRE is incubated with cofactor-NR complexes on beads in protein-DNA binding buffer for 1 h at room temperature in protein-DNA binding buffer.

procedure, we are able to screen a large number of compounds within 4 h.

We previously showed that PCB suppresses TR-mediated transcription (4,5), which is a critical step for normal brain development (10,11). Using EMSA, we showed that PCB suppression of TR-mediated transcription is due to

partial dissociation of TR from TRE (5). Based on this finding, we tested our novel assay using GST-TRβ1 and DIG-labeled TRE (Figure 2A). GST did not interact with DIG-TRE (Figure 2A, columns 1 and 2), whereas GST-TRβ1 showed binding in the absence of ligand (Figure 2A, column 3). Adding triiodothyronine

Figure 2. Examples of the liquid chemiluminescent DNA pull-down assay. (A–C) Light emission was measured from the liquid chemiluminescent DNA pull-down assay to indicate binding. The histograms indicate relative light emission. Data are shown as means $\pm$ SEM. (A) Effect of polychlorinated biphenyl (PCB) on thyroid hormone receptor β 1 (TR β 1)-thyroid hormone response element (TRE) binding. Glutathione *S*-transferase (GST)-TR β 1 was incubated with digoxigenin (DIG)-labeled TRE with or without T3 and/or PCB. (B) Effect of PCB on steroid receptor coactivator-1 (SRC-1)-TR β 1-TRE binding. GST-SRC-1 was incubated with in vitro-translated TR β 1 and retinoid X receptor (RXR), then SRC-1-TR β 1/RXR complex was incubated with DIG-labeled TRE with or without T3 and/or PCB for 1 h at room temperature. (C) Effect of tamoxifen on silent mediator for retinoid and thyroid hormone (SMRT)-estrogen receptor α (ER α)-estrogen response element (ERE) binding. GST-SMRT was incubated with ER α , then SMRT-ER α complex was incubated with DIG-labeled ERE with or without 17 β -estradiol (E2) and/or tamoxifen (TAM). After detection reaction, light emission was measured to indicate binding as described above. Data are shown as means $\pm$ SEM.

(T3) reduced binding (Figure 2A, column 5), and PCB dissociated TR from TRE in the absence or presence of T3 (Figure 2A, columns 4 and 6). These are consistent with the findings observed with EMSA (5).

Since NR-mediated transcription is not exerted directly by NR itself but by cofactors, we further developed the assay to detect cofactor-NR-NRE binding (Figure 1B, 2). In vitro-translated NR is prepared using rabbit reticulocyte lysate (Promega, Madison, WI, USA) according to the manufacturer's instruction using 1 μ g expression plasmid in a final reaction volume of 50 μ L. GST-fused cofactor (~2 μ g/reaction) on glutathione-Sepharose beads is incubated with 5 μ L in vitro-translated NR in protein-protein binding buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Triton X-100, 10% glycerol, 1 mM DTT) in a final volume of 1 mL for 1 h at room temperature or 16 h at 4°C. Thereafter, 10–20 fmol DIG-labeled double-stranded DNA fragments containing an NRE are incubated with the GST-cofactor-NR complex on glutathione-Sepharose beads in protein-DNA binding buffer from the kit for 1 h at room temperature. During

this incubation, GST-fused cofactor on the beads binds to in vitro-translated NR that could bind DIG-labeled DNA. The order of incubation for protein-DNA binding and protein-protein binding can be changed. Subsequent steps are the same as described above in the assay to detect NR-NRE binding.

We reported that TR β 1/retinoid X receptor (RXR) binds to TRE in the absence or presence of T3 (5). Adding T3 caused TR β 1/RXR complexes to bind steroid receptor coactivator-1 (SRC-1) in a T3-dependent manner (5) (Figure 2B, columns 4 and 6). PCBs do not affect TR β 1-SRC-1 binding (5). However, adding PCB dissociated about half of the TR β 1/RXR-SRC-1 complex from TRE/RXR (Figure 2B, column 7). This result is comparable to those of functional data (4,5) and EMSA (5).

We also used estrogen receptor α (ER α) and estrogen response element (ERE) (Figure 2C). In the absence of ligand, ER α bound to ERE. The interaction between ER α and silent mediator for retinoid and thyroid hormone (SMRT) was limited (Figure 2C, column 3). Adding tamoxifen induced ER α and SMRT binding as reported (Figure 2C, column 4). These results are comparable

to those obtained using GST pull-down assays (2).

Our novel technique generates simultaneous results for large numbers of compounds, consistent with findings obtained with EMSA, but within 4 h and with minimal waste and opportunity for contamination. In addition, this assay can detect subtle binding that could become dissociated during migration through acrylamide gels. This technique can be used to examine the binding between most DNA binding proteins and DNA in vitro. We have also applied this technique to RNA-protein binding and protein-protein binding (data not shown). The results showed that our assay was versatile and sensitive enough to discriminate binding, which can be difficult to detect by EMSA or GST pull-down assays.

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COMPETING INTERESTS STATEMENTS

T.I. was previously employed by Eli Lilly and Company. The other authors have no competing interests to declare.

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