A Simple ELISA for Screening Ligands of Peroxisome Proliferator-activated Receptor γ

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Peroxisome proliferator-activated receptors (PPARs) are orphan nuclear hormone receptors that are known to control the expression of genes that are involved in lipid homeostasis and energy balance. PPARs activate gene transcription in response to a variety of compounds, including hypolipidemic drugs. Most of these compounds have high affinity to the ligand-binding domain (LBD) of PPARs and cause a conformational change within PPARs. As a result, the receptor is converted to an activated mode that promotes the recruitment of co-activators such as the steroid receptor co-activator-1 (SRC-1). Based on the activation mechanism of PPARs (the ligand binding to PPARγ induces interactions of the receptor with transcriptional co-activators), we performed Western blot and ELISA. These showed that the indomethacin, a PPARγ ligand, increased the binding between PPARγ and SRC-1 in a ligand dose-dependent manner. These results suggested that the *in vitro* conformational change of PPARγ by ligands was also induced, and increased the levels of the ligand-dependent interaction with SRC-1. Collectively, we developed a novel and useful ELISA system for the mass screening of PPARγ ligands. This screening system (based on the interaction between PPARγ and SRC-1) may be a promising system in the development of drugs for metabolic disorders.

Keywords: Enzyme-linked immunosorbent assay, Glutathione S-transferase, Peroxisome proliferator-activated receptor-γ2, Steroid receptor coactivator-1

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Introduction

Orphan nuclear receptors provide multi-cellular organisms with a means to directly control the gene expression in response to a wide range of development, physiological, and environmental cues. The activity of the orphan nuclear receptor is controlled by at least three distinct mechanisms. One is a binding of a small lipophilic ligand by the receptor or its partner in heterodimer complexes. Another is a covalent modification, usually in the form of phosphorylation that is regulated by events at the cellular membrane or during the cell cycle. The other is a protein-protein interaction, generally through contacts with other transcription factors, including nuclear receptors themselves (Giguere *et al.*, 1999). The activity of nuclear receptors could be potentially regulated by natural ligands. Their ligands play crucial roles in development, homeostasis, and disease. In the past few years, the PPAR family of the orphan nuclear receptor has been intensively investigated. The nuclear receptors regulate energy balance and hormone response and change of energy balance toward an excessive energy intake that is associated with a number of prevalent metabolic disorders, such as obesity, artherosclerosis, and type 2 diabetes.

The PPARs consist mainly of three subtypes (PPARα, PPARδ/β, and PPARγ). These subtypes have a similar structural organization and sequence homology. The ligand independent activation region (N-terminal region) can confer constitutive activity on the receptor (Werman *et al.*, 1997) and is negatively regulated by phosphorylation (Shao *et al.*, 1998). This region is followed by a DNA-binding region that consists of two zinc fingers that are separated by a linker region and ligand binding region (C-terminal). However, it is clear that these receptors possess distinct functions. PPARα is expressed predominantly in the liver, and involved in peroxisome proliferation and regulation of fatty acid catabolism (Issemann *et al.*, 1993). PPARδ/β is expressed in most cell types; however, its role

remains unclear (Schmidt *et al.*, 1992). PPARγ has at least two promoters, resulting in the production of two isoforms, 1, 2. These isoforms are expressed in a tissue-specific pattern. The PPARγ1 isoform is expressed in the spleen, intestine, and white adipose tissue, while the PPARγ2 is preferentially expressed in white and brown fat. PPARγ2 is most abundantly expressed in the fat cell, and plays a pivotal role in fat cell differentiation and lipid storage (Tontonoz *et al.*, 1994). The distinct physiological role of each subtype has been shown to bind a discrete set of ligands. Although fatty acids could activate the PPARs, PPARα activity was induced by eicosanoids (Yu *et al.*, 1995), carbaprostacyclin (Hertz *et al.*, 1996), and non-steroidal anti-inflammatory drugs (NSAIDs) (Lehmann *et al.*, 1997). PPARδ/β is activated by several polyunsaturated fatty acids (Schmidt *et al.*, 1992) and eicosanoids (Forman *et al.*, 1997). PPARγ specifically binds to thiazolidinediones (TZDs), a class of antidiabetic drugs. Other PPARγ ligands include the natural prostaglandin metabolite 15-deoxy-$\Delta^{12.14}$-prostaglandin J_2 (PGJ$_2$), polyunsaturated fatty acids, and NSAIDs such as ibuprofen and indomethacin.

PPARγ specific ligands (PPARγ agonists) were recently reported to have new therapeutic roles. PPARγ ligands inhibit the activation of macrophage and monocyte, suppress tumor cell growth (Elstner *et al.*, 1998), inhibit angiogenesis *in vitro* and *in vivo* (Xin *et al.*, 1999), and induce terminal differentiation of human liposarcoma cells (Tontonoz *et al.*, 1997). The specific ligands of PPARγ have high affinity to the ligand-binding domain of PPARγ and cause a conformational change within PPARγ. As a result, the receptor is converted to an activated mode that promotes the recruitment of co-activators and effectively stimulates the transcription of the gene-associated energy balance. It is dependent on allosteric alterations in the AF-2 helical domain (Horwitz *et al.*, 1996). These co-activators are the p160 proteins, which are identified as members of a gene family of steroid receptor co-activating factors, referred to as SRC-1/NcoA-1 (Zhu *et al*, 1996), TIF/GRIP-NcoA-2 (Voegel *et al.,* 1996), and pCIP/ACTR/AIB1 (Torchia *et al.*, 1997). Different ligands of PPARs may elicit distinct downstream biological effects, because of the unique conformational changes in the nuclear receptor. The nuclear receptor-interaction domain of p160 proteins is highly conserved, and contains three or four repeated motifs of the consensus sequence LXXLL.

The *in vitro* screening system for the detection of PPAR ligands may assist in developing drugs for clinical diseases. In our report, we established the *in vitro* screening system, based on the ligand-dependent binding between PPARγ2 and SRC-1. The ELISA system was adapted for more conventional and rapid screening of natural materials, such as phyto-chemical and various synthetic candidate ligands. Therefore, in order to gain a major technical advantage, there is no requirement for radioactive labeling of candidate ligands, and it is sensitive. This simple screening system could be available for the development of potential drugs against obesity, diabetes, inflammatory diseases, and cancer.

Materials and Methods

RT-PCR and recombinant plasmid DNAs

Preparation of recombinant human PPARγ2 and mouse PPARγ2 - Total RNA was isolated from human lipomas and the differentiated 3T3-L1 cell (day 10) by acidic phenol-guanidinium thiocyanate-chloroform extraction (Sambrook *et al.*, 1989). The human PPARγ2 and mouse PPARγ were amplified by RT-PCR (ProSTAR™, Stratagene, LaJolla, USA), as described (Lee *et al.,* 2000), using the same primer sets that comprised 5'-GCG TCG ACT CAT GGG TGA AAC TCT GG -3' (sense) and 5'-CCG CTC GAG CTA ATA CAA GTC CTT GT-3' (antisense). PCR products were double digested by the restriction enzymes of both *Sal* I and *Xho* I. The PPARγ2 fragment was cloned into pGEX4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden) to construct GST-fused PPARγ2. Transformations of pGEX4T-1/PPARγ2 into *E. coli* DH5α (Pharmacia, Les Ulis, France) were performed to express GST-PPARγ2. The PPARγ2 N-terminal region (1-255 a.a) insert was amplified by the same methods and used primer sets that were comprised of 5'-GCG TCG ACT CAT GGG TGA AAC TCT GG-3' (sense) and 5'-ATA AGA ATG CGG CCG CCT ACG GGA AGG A-3' (anti-sense). The RCR product was double digested by the restriction enzymes of both *Sal* I and *Not* I. The PPARγ2 N-terminal region (1-255 a.a) fragment was cloned into the pGEX4T-1 to construct the GST-fused PPARγ2 N-terminal region. Transformations of the pGEX4T-1/ PPARγ2 N-terminal region into *E. coli* DH5α were performed to express the GST-PPARγ2 N-terminal region.

Preparation of recombinant SRC-1 - Total RNA was isolated from monolayers of differentiated 3T3-L1 (day 10) by acidic phenol-guanidinium thiocyanate-chloroform extraction. The SRC-1 (633-783 a.a) insert was amplified by RT-PCR, using primer sets that were comprised of 5'-CCG GAA TTC AAG CCT CTG GAC TCA GGA C-3' (sense) and 5'-CCC AAG CTT TTG GGT TTG TGT TAC AAG-3' (antisense). The PCR product was double digested by the restriction enzymes of both *Eco*R I and *Hind* III. An SRC-1 fragment was cloned into pET28a (+) (Novagen, Madison, USA) to construct 6xHis tagged SRC-1. Transformations of pET28a/SRC-1 into *E. coli* BL21 (DE3) pLys S (Pharmacia, Les Ulis, France) were performed to express 6xHis-SRC-1.

Over-expression of PPARγ2 and SRC-1 *E. coli* that harbored pGEX4T-1/human PPARγ2 or pGEX4T-1/mouse PPARγ2 were grown, induced for protein expression overnight at 20°C with 1 mM isoprophyl-β-D-thiogalactopyranoside (IPTG), harvested, and lysed in a phosphate-buffered saline (PBS) that contained 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10μg/ml of aprotinin, and sonicated. The resulting mixture was centrifuged for 30 min at 12,000 rpm in order to remove the pellets that contained the cell debris. The supernatants were used as cell lysates for the binding assay. The protein that was expressed in the pET28a vectors was prepared in *E. coli* BL21 (DE3) Lys S using the same procedure as previously described, except for using a lysis buffer that contained 50 mM NaH$_2$PO$_4$ and 150 mM NaCl, pH 8.0. The recombinant SRC-1 protein, which contained 6xHis residues at the N-terminus, was purified by Ni^{2} affinity chromatography under native conditions (Park *et al.,* 2000; Cho *et al.,* 2001). After the
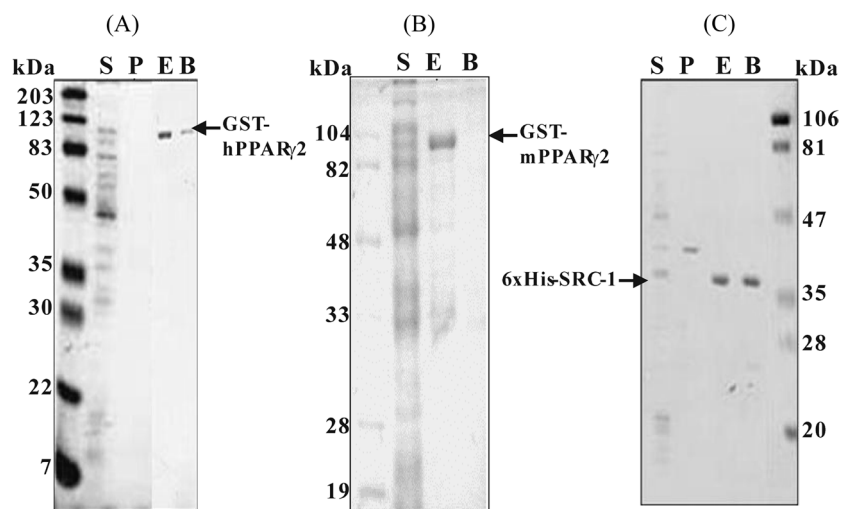
Fig. 1. Over-expression of GST-fused human PPARγ2 (A), GST-fused mouse PPARγ2 (B), and His-tagged mouse SRC-1 (C). The *E.coli* lysates after sonication were separated into supernatant and pellet by centrifugation at 12,000 rpm for 30 min. Diluted aliquots (1/10) of each fraction were analyzed on 12% SDS-PAGE electrophoresis and stained with Coomassie blue. GST-fused PPARγ2 was purified with GSH-Sepharose. His-tagged SRC-1 was purified with a Ni^{+2}-NTA column. S, supernatant; P, pellet; E, elutant; B, bead.

6xHis protein was loaded onto the Ni^{+2}-NTA columns (Peptron, Taejon, Korea), the column was washed twice with a wash buffer (20 mM imidazole, 50 mM Na_2HPO_4, 300 mM NaCl, pH 8.0), followed by elution with an elution buffer (200 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The purified 6xHis-SRC-1 was then aliquotted and stored at −72°C until use. Concentrations of the proteins were determined by a Bradford reagent (Bio-Rad Lab., Hercules, USA)

Western blot and *in vitro* binding assay for SRC-1 and PPARγ
The purified GST-PPARγ2 protein was examined with an SDS-polyacrylamide gel and Western blot assay. The gels were transferred to Immobilon-P membranes (Millipore, Bedford, USA) at 50 V for 1.5 h at room temperature. The membranes were blocked by 5% skimmed milk. The membrane was probed with an anti-PPARγ2 antibody (Calbiochem, San Diego, USA) diluted 1 : 2000 in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20), and followed by an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, St. Louis, USA). Visualization was achieved with a NBT/BCIP substrate kit (Bio-Rad Lab). To identify the binding between PPARγ2 and SRC-1, 0.4 mg/ml of the purified 6xHis-SRC-1 was used in a SDS-polyacrylamide gel electrophoresis. The gel was transferred to Immobilon-P membranes at 50 V for 1.5 h at room temperature. The membrane was blocked by 5% skimmed milk. A TBST buffer that contained 0.4 mg/ml of purified GST-PPARγ2 was added and incubated for 1 h at room temperature. The membrane was probed with an anti-PPARγ2 antibody, followed by an alkaline phosphatase-conjugated anti-rabbit IgG. Visualization was achieved with a NBT/BCIP substrate kit.

Optimization of ELISA The 6xHis-SRC-1 recombinant protein that was bound to beads was dissociated with an elution buffer (200 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). A Maxisorb 96-well plate (Nunc, Roskilde, Denmark) was coated with the eluted SRC-1 protein at a dose of 8 μg/ml. Serial dilutions of the GST-PPARγ2 lysate were added to the SRC-1 pre-coated plate. Then serial dilutions of the indomethacin (Sigma), BADGE (bisphenol A 2, 3-dihydroxypropyl glycidyl ether; Fluka Chemika, Buchs, Switzerland), TZD moiety (2,4-thiazolidinedione; Aldrich Chem. Co., St. Louis, USA), and Wy14,643 (ChemSyn Laboratories, Lenexa, USA) were added. The mixtures were allowed to incubate for 1 h at room temperature. After exhaustive washing with PBS that contained 0.05% Tween-20 (PBST) in order to remove the unbound GST-PPARγ2 protein, the rabbit anti-GST antibody (Molecular Probes, Eugene, USA) diluted 1 : 3,000 in PBST was dispensed to the plate, followed by a 1 h incubation. After washing, the horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma) was applied to the plate for an immunoassay. After washing with PBST, 100 μl of the substrate (4 mg *o*-phenylene diamine, 5 μl 37% H_2O_2 per 10 ml of 0.1M citrate buffer, pH 5.1) was added. The enzyme activity was detected at 490 nm using an ELISA reader (Molecular Devices, Hercules, USA), after stopping the enzyme reaction with 2.5 N sulfuric acid.

Results and Discussion

Over-expression and purification of recombinant proteins PPARγ2 and SRC-1 In attempt to apply the physical interaction of PPARγ2 with the SRC-1 protein for the screening system, the PPARγ2 cDNA was primarily cloned into the bacterial expression vector pGEX4T-1. The recombinant vector was transformed into *E. coli* DH5α competent cells for the induction of corresponding-fused proteins. The GST-fused human PPARγ2 (Fig. 1A) and mouse PPARγ2 protein (Fig. 1B) (used for *in vitro* binding assay and ELISA) were over-expressed (Fig. 1). The proteins that were present in both the pellet and the supernatant fractions after
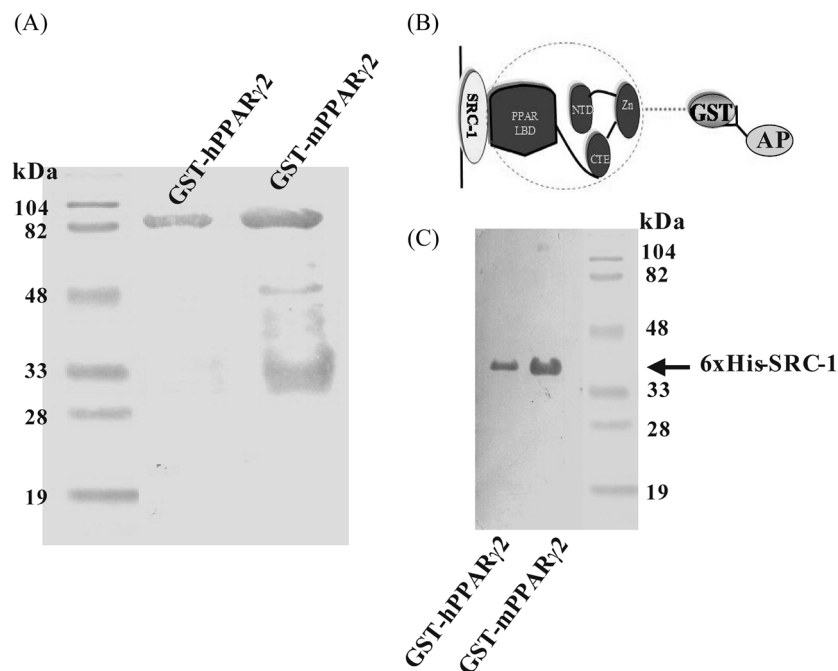
Fig. 2. Characterization of recombinant proteins including GST-fused human PPARγ2, mouse PPARγ2, and 6xHis-tagged mouse SRC-1 by Western blot analysis. For Western blotting, GST-fused human PPARγ2 and GST-fused mouse PPARγ2 were separated on 12% SDS-PAGE, blotted on the Immobilon-P membrane, and probed by the anti-PPARγ antibody (A). In order to test the binding between PPARγ2 and SRC-1, the purified His-tagged SRC-1 protein was loaded on the 12% SDS-PAGE gel and transferred on the Immobilon-P membrane. This was followed by adding the *E coli* lysate that harbors the human PPARγ2 or mouse PPARγ2 protein. The binding between PPARγ2 and SRC-1 was identified by using an anti-PPARγ antibody (C). (B) is a scheme of binding between PPARγ2 and SRC-1 in a Western blot.

lysis were resolved by SDS-PAGE and identified by a Western blot using an anti-PPARγ2 antibody (Fig. 2A). The GST-PPARγ2 protein was clearly visible at the predicted molecular weight, migrating at approximately 82 kDa. A considerable amount of GST-fused PPARγ2 protein was present in the supernatant, as determined by the SDS-PAGE. The SRC-1 was cloned into the bacterial expression vector pET28a in order to express the 6xHis-tagged form. The recombinant vector was transformed into *E. coli* BL21 (DE3) competent cells for the induction of corresponding-tagged proteins. Its expression levels in both the supernatant and the pellet fractions were analyzed by SDS-PAGE. The SDS-PAGE electrophoresis showed that many of the SRC-1 proteins that migrated at approximately 36kDa were present as a soluble form in the supernatant fraction (Fig. 1C).

Binding assay between PPARγ2 and SRC-1 by immunoblot
As a preliminary experiment for the PPARγ2 and SRC-1 interaction, 80 μg of the purified SRC-1 protein was loaded onto the 12% SDS-PAGE gel, electrophoresed, and transferred to the PVDF membrane. The PVDF membrane that contained the SRC-1 protein was incubated with the *E.coli* lysate that contained the PPARγ2 proteins. After an extensive washing, the PPARγ that was bound to SRC-1 was analyzed by immunoblotting using an anti-PPARγ antibody

(Fig. 2B and C). The SRC-1 protein was detected in about the 36kDa position by PPARγ2, as expected. This result revealed that SRC-1 was bound to PPARγ2 *in vitro*.

Optimization of ELISA for screening PPARγ agonists
The PPARγ-RXRα heterodimers can be activated by the ligands of PPARγ or RXRα. This ligand-dependent transcriptional activity responds to carboxyl-terminal helical regions (activation function, AF2) (Durand *et al.*, 1994). The AF-2 domain forms part of the ligand-binding pocket and undergoes a conformational change that is required for the recruitment of the co-activator proteins. These proteins include NcoA-1/SRC-1. The LXXLL motif of SRC-1 contacts LBD (ligand-binding domain) of PPARγ in the presence of the PPARγ agonist, which results in the displacement of the PPARγ AF-2 domain. This allows the binding of the RXRα ligands to RXRα, which promotes the binding of the second LXXLL motif from the same SRC-1 molecule. These results may explain why the PPARγ-specific ligands can provide different responses to the nuclear-receptor heterodimers. The multicomplex of the ligand-bound heterodimer co-activator recruits other co-regulators (P/CIP, CBP, P/CAF, etc.) (Zhu *et al.*, 1996; Torchia *et al.*, 1997; Chen *et al.*, 2000). This multicomplex transcriptional co-regulator triggers the RNA polymerase machinery in the
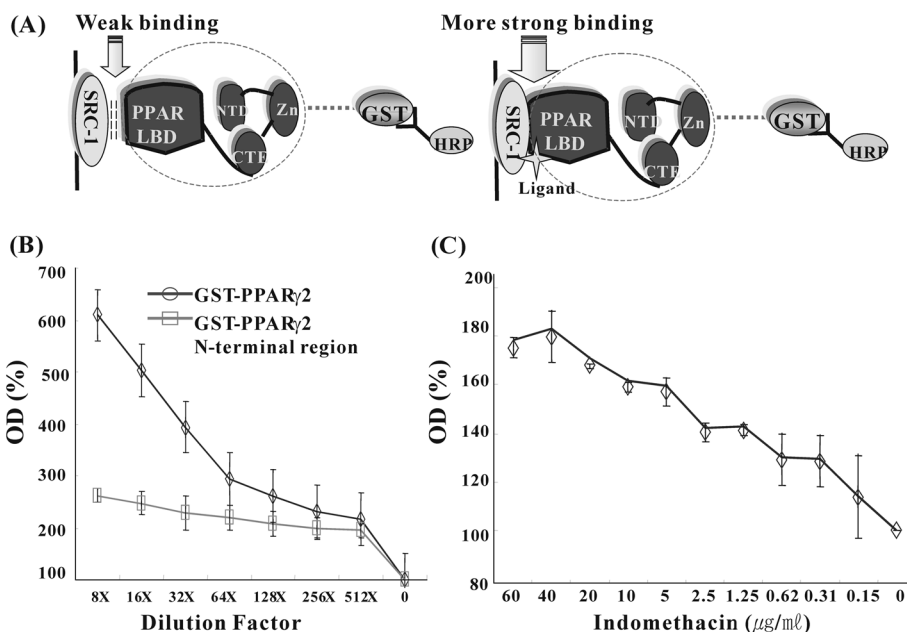
Fig. 3. Optimization of ELISA based on the binding between SRC-1 and PPARγ2. The purified SRC-1 protein was coated onto a Maxisorb 96 well plate, and serial dilutions of lysates that contained GST-PPARγ2 were added with or without various concentrations of indomethacin. The PPARγ2 that bound to SRC-1 was detected with a rabbit monoclonal anti-GST antibody, followed by incubation of a horseradish peroxidase-conjugated anti-rabbit IgG. Bound-enzyme activity was detected by a ELISA reader (A). The specific binding between SRC-1 and GST-PPARγ2. As a nonspecific control, the GST-N-terminus domain of PPARγ2 was added into SRC-1 coated wells and detected with a rabbit monoclonal anti-GST antibody, as previously described (B). The effect of the ligand on the binding between PPARγ2 and SRC-1. The 128 times-diluted *E.coli* lysate that harbors GST-PPARγ2 was added to the SRC-1 coated wells with increasing concentrations of indomethacin, a PPAR agonist (C). Results are the mean ± standard deviation and are representative of the three experiments.

target that is downstream of the PPRE gene (PPAR response elements). The PPARγ target genes with their response elements are genes that are associated with lipid metabolism and hormone homeostasis (Baumann *et al.*, 2000).

In previous reports, it was asserted that the ligand would also change the conformation of PPARγ and enhance the binding of PPARγ-SRC-1 *in vitro* (Tolon *et al.*, 1998). This concept was applied to the enzyme-linked immunosorbent assay (ELISA) (Lee *et al.*, 2001) to screen the PPARγ ligand. The purified SRC-1 was plated on the 96 wells. The *E.coli* lysate that contained the GST-fused PPARγ2 and putative ligands were added into the SRC-1 coated wells. The binding of SRC-1 and PPARγ2 was enhanced by some ligands, and the binding was detected by the anti-GST antibody-conjugated horseradish peroxidase (Fig. 3). This ELISA system could be used to screen a potential PPARγ ligand.

In order to test the binding between SRC-1 and PPARγ2 in the ELISA plate, 8 μg/ml of His-tagged SRC-1 was pre-coated. Serial dilutions of the *E.coli* lysate that contained GST-fused PPARγ2 were added and incubated for 1 h. The GST-fused PPARγ2 that was bound to SRC-1 was detected with a rabbit monoclonal anti-GST antibody, followed by incubation of a horseradish phosphatase-conjugated anti-rabbit IgG. After washing, the bound enzyme activity was detected at 490 nm using an ELISA reader. The binding

between the SRC-1 protein and PPARγ2 protein was detected *in vitro*. The binding was dose dependent (Fig. 3B). The PPARγ ligands bind to the LBD, which is located at the C-terminal region of the PPARγ, then the major activity of PPARγ2 is transcriptionally initiated. The PPARγ LBD is bound to the LXXLL motif of SRC-1 and complexes hetero-dimer with SRC-1. However, the N-terminal domain (modulation region) of PPARγ2 has little binding to SRC-1 (Fig. 3B). In addition, we tried to enhance the PPARγ2-SRC-1 binding by using ligands. The His-tagged SRC-1 was pre-coated with a concentration of 8 μg/ml (both of the 128 times-diluted *E.coli* lysate that contained GST-fused PPARγ2 and various concentrations of indomethacin, a PPARγ ligand) (Fig. 3C). The binding experiments, using various concentrations of ligands, were repeated more than three times. These data suggest that the binding of PPARγ-SRC-1 was dose dependent to their ligands *in vitro*.

Application of ELISA system based on PPARγ-SRC-1 binding In order to evaluate the ELISA system, based on the binding between PPARγ and SRC-1, the PPAR agonists (indomethacin, WY14,643), antagonist (BADGE), and mal-functional ligand (TZD moiety) were used (Fig. 4). Various ligands were elucidated to see if they could increase the binding between SRC-1 and human PPARγ2 (Fig. 4A), and
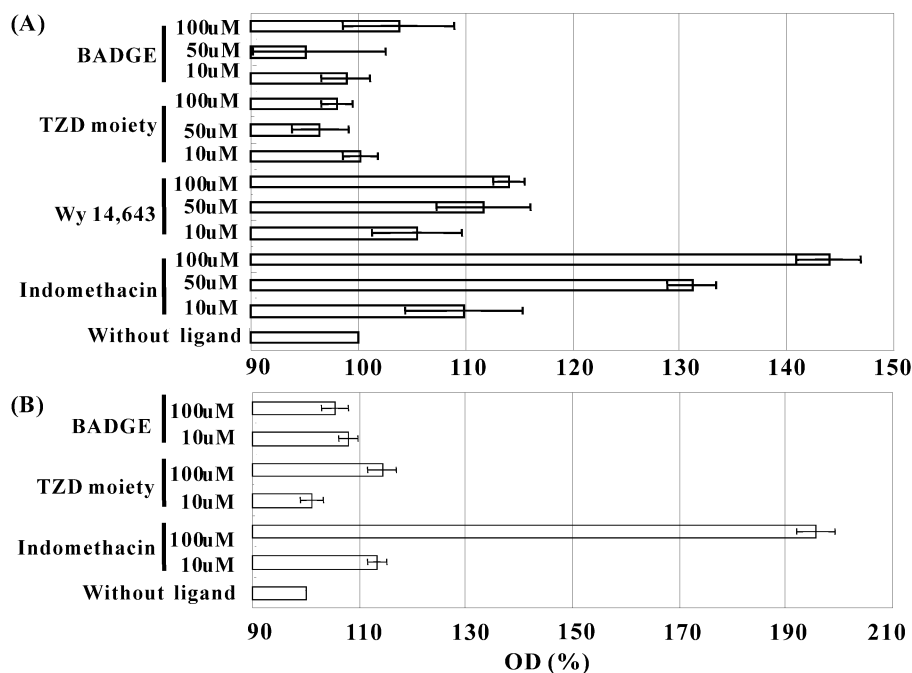
Fig. 4. ELISAs based on binding between human PPARγ2 and SRC-1 for screening various PPAR ligands (A) and between mouse PPARγ2 and SRC-1 (B). The PPAR agonist (indomethacin, Wy 14,643), mal-functional ligand (TZD moiety), and antagonist (BADGE) were treated in the ELISA system.

modulate the binding between SRC-1 and mouse PPARγ2 (Fig. 4B). These data showed that PPAR ligands, such as indomethacin and WY 14,643, elevated the affinity of the PPARγ-SRC-1 binding, while the mal-functional ligand, TZD moiety, and antagonist, BADGE, had no affect. Unexpectedly, the PPARγ antagonist (BADGE) was unable to decrease the PPARγ-SRC-1 binding. Therefore, we suggest that our ELISA system is a useful system for screening potential drugs that target PPAR agonists, but not the PPAR antagonist. The ELISA system, based on the interaction between PPARγ2SRC-1, has been successfully used to characterize the function of various ligands. It is a novel and reliable assay system to detect the PPAR agonists, but not the antagonist. This approach may be applied to nuclear receptors with or without known ligands, because the SRC-1 fragment interacts with a broad range of nuclear receptors. Compared to the previous ligand-binding assay (Krey *et al.*, 1997; Elbrecht *et al.*, 1999; Zhou *et al.*, 2001), our ELISA screen system has many technical advantages: (1) It does not require radioactive labeling of the candidate ligands or proteins. (2) It does not use a pull-down assay, which takes time to perform. (3) It is very simple to simultaneously screen a large number of compounds. In addition, the fact that this screening system is based on a physical-functional interaction between two proteins in response to ligand, and not merely on the association of a lipophilic molecule with a globular protein domain, suggests that it should be less prone to artifacts. However, the assay depends on a soluble GST-PPAR fusion protein, which cannot be taken for granted.

It was recently reported that PPARγ plays new roles in the activation of macrophage and monocyte, and tumor cell growth. This function is also associated with their specific ligands. Therefore, this screening system may contribute to the discovery of new therapeutic agents against obesity, type2 diabetes, and inflammatory diseases.

References

Baumann, C. A., Chokshi, N., Saltiel, A. R. and Ribon, V. (2000) Cloning and characterization of a functional peroxisome proliferator activator receptor-gamma-responsive element in the promoter of the CAP gene. *J. Biol. Chem.* **275**, 9131-9135.

Chen, S., Johnson, B. A., Li, Y., Aster, S., McKeever, B., Mosley, R., Moller, D. E. and Zhou, G. (2000) Both co-activator LXXLL motif-dependent and -independent interactions are required for peroxisome proliferator-activated receptor gamma (PPARgamma) function. *J. Biol. Chem.* **275**, 3733-3736.

Cho, Y. S., Cho, C. W., Kang, J. W., Cho, M. C., Lee, K. A., Shim, J. H., Kwon, D. H., Choe, Y. K., Park, S. N. and Yoon, D. Y. (2001) Development of a screening system for drugs against human papillomavirus-associated cervical cancer: Based on E7-Rb binding. *J. Biochem. Mol. Biol.* **34**, 80-84.

Durand, B., Saunders, M., Gaudon, C., Roy, B., Losson, R. and

Chambon, P. (1994) Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. *EMBO. J.* **13**, 5370-5382.

Elbrecht, A., Chen, Y., Adams, A., Berger, J., Griffin, P., Klatt, T., Zhang, B., Menke, J., Zhou, G., Smith, R. G. and Moller, D. E. (1999) L-764406 is a partial agonist of human peroxisome proliferator-activated receptor. *J. Biol. Chem.* **274**, 7913-7922.

Elstner, E., Muller, C., Koshizuka, K., Williamson, E. A., Park, D., Asou, H., Shintaku, P., Said, J, W., Heber, D. and Koeffler, H. P. (1998) Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *Proc. Natl. Acad. Sci. USA* **95**, 8806-8811.

Forman, B. M., Chen, J. and Evans, R. M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc. Natl. Acad. Sci. USA* **94**, 4312-4317.

Giguere, V. (1999) Orphan Nuclear Receptors: From Gene to Function. *Endocr. Rev.* **20**, 689-725.

Hertz, R., Berman, I., Keppler, D. and Bar-Tana, J. (1996) Activation of gene transcription by prostacyclin analogues is mediated by the peroxisome-proliferators-activated receptor (PPAR). *Eur. J. Biochem.* **235**, 242-247.

Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S. and Tung, L. (1996) Nuclear receptor co-activators and co-repressors. *Mol. Endocrinol.* **10**, 1167-77.

Issemann, I., Prince, R. A., Tugwood, J. D. and Green, S. (1993) The peroxisome proliferator-activated receptor:retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipidaemic drugs. *J. Mol. Endocrinol.* **11**, 37-47.

Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M. G. and Wahli, W. (1997) Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by co-activator-dependent receptor ligand assay. *Mol. Endocrinol.* **11**, 779-791.

Lee, I. H., Kim, I. S. and Lee, S. K. (2000) Expression of all-trans retinoic acid glycoprotein and inflammatory cytokines during differentiation of HL-60 cell. *J. Biochem. Mol. Biol.* **33**, 402-406.

Lee, S. S., Lee, K. L., Yoo, J. Y., Jeong, M. J., Park, Y. M., Kwon, B. M., Bae, Y. S. and Han, M. Y. (2001) Development of ELISA system for screening of specific binding inhibitors for Src homology(SH)2 domain and phosphotyrosine interactions. *J. Biochem. Mol. Biol.* **34**, 537-543.

Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M. and Kliewer, S. A. (1997) Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **272**, 3406-3410.

Park, J. S., Lee, H. G., Lee, Y., Kang, Y. H., Rhim, H. S. and Choi, S. Y. (2000) Expression of human immunodeficiency virus type 1 Tat proteins in *Escherichia coli* and application to study Tat functions. *J. Biochem. Mol. Biol.* **33**, 337-343.

Sambrook, J. Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, New York.

Schmidt, A., Endo, N., Rutledge, S. J., Vogel, R., Shinar, D. and Rodan, G. A. (1992) Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol. Endocrinol.* **6**, 1634-1641.

Shao, D., Rangwala, S. M., Bailey, S. T., Krakow, S. L., Reginato, M. J. and Lazar, M. A. (1998) Interdomain communication regulating ligand binding by PPAR-gamma. *Nature* 396, 377-380.

Tolon, R. M., Castillo, A. I. and Aranda, A. (1998) Activation of the prolactin gene by peroxisome proliferator-activated receptor-alpha appears to be DNA binding-independent. *J. Biol. Chem.* **273**, 26652-26661.

Tontonoz, P., Hu, E., Graves, R, A., Budavari, A. I. and Spiegelman, B. M. (1994) mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* **8**, 1224-1234.

Tontonoz, P., Singer, S., Forman, B. M., Sarraf, P., Fletcher, J. A., Fletcher, C. D., Brun, R. P., Mueller, E., Altiok, S., Oppenheim, H., Evans, R. M. and Spiegelman, B. M. (1997) Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. *Proc. Natl. Acad. Sci. USA* **94**, 237-241.

Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K. and Rosenfeld, M. G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387**, 677-684.

Voegel, J. J., Heine, M. J., Zechel, C., Chambon, P. and Gronemeyer, H. (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO. J.* **15**, 3667-3675.

Werman, A., Hollenberg, A., Solanes, G., Bjorbaek, C., Vidal-Puig A. J. and Flier, J. S. (1997) Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor gamma (PPARgamma). *J. Biol. Chem.* **272**, 20230-20235

Xin, X., Yang, S., Kowalski, J. and Gerritsen, M. E. (1999) Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis *in vitro* and *in vivo*. *J. Biol. Chem.* **274**, 9116-9121.

Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M. and Lazar, M. A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J. Biol. Chem.* **270**, 23975-23983.

Zhou, G., Cummings, R., Hermes, J. and Moller, D. E. (2001) Use of homogeneous time-resolved fluorescence energy transfer in the measurement of nuclear receptor activation. *Methods* **25**, 54-61.

Zhu, Y., Qi, C., Calandra, C., Rao, M. S. and Reddy, J. K. (1996) Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a co-activator of peroxisome proliferator-activated receptor gamma. *Gene Expr.* **6**, 185-195.