Development of a Novel PPARγ Ligand Screening System Using Pinpoint Fluorescence-Probed Protein

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The activation of peroxisome-proliferator-activated receptor-γ (PPARγ), which plays a central role in adipocyte differentiation, depends on ligand-dependent co-activator recruitment. In this study, we developed a novel method of PPARγ ligand screening by measuring the increase in fluorescent polarization accompanied by the interaction of a fluorescent co-activator and PPARγ. Sterol receptor co-activator-1 (SRC-1), a major PPARγ co-activator, was probed by fluorescent TAMRA by the Amber codon fluorescence-probe method. Polarization was increased by adding PPARγ ligands to a solution containing labeled SRC-1 (designated TAMRA-SRC-S) and PPARγ. The disassociation constants (Kd) of the PPARγ-synthesized ligands, pioglitazone (221 nm), troglitazone (83.0 nm), and 15-deoxy-12,14-prostaglandin J2 (15d-ΔPGJ2) (156 nm), were determined by this method. Farnesol (2.89 μM) and bixin (21.1 μM), which we have reported to be PPARγ ligands, increased the fluorescent polarization. Their Kd values were in agreement with the ED50 values obtained in the luciferase assay. The results indicate that the method is valuable for screening natural PPARγ ligands.

Key words: peroxisome-proliferator-activated receptor-γ (PPARγ); co-activator; high-throughput; ligand; screening system

Peroxisome-proliferator-activated receptor-γ (PPARγ), a member of the nuclear hormone receptor superfamily, plays a central role in the regulation of adipocyte differentiation.1) Thiazolidinediones (TZDs), major insulin sensitzers, promote adipocyte differentiation through PPARγ activation2) to increase the number of small adipocytes with high insulin sensitivity and to decrease the number of large adipocytes with insulin resistance.3) Thus, TZDs improve metabolic disorders such as systemic insulin resistance. As PPARγ is a valuable target for the treatment of diabetes and hyperlipidemia, research and development of PPARγ ligands has been aggressively performed.3) Among the many PPAR ligand screening methods, luciferase reporter assay is a major, effective method.4–7) It is based on the ligand-dependent modulation of nuclear-receptor-mediated transcription. Using it, antagonists can also be detected. We and other groups have found several endogenous and natural ligands by this method. However, including other screening methods, such as the use of the Biacore system and ELISA, currently available methods of PPARγ ligand screening require complex procedures such as cell culture and protein fixation. Therefore, a novel high-throughput system is desired for easier PPARγ ligand screening.

Nuclear-receptor mediated transcriptional regulation, including PPARγ, depends on co-activator binding to nuclear receptors induced by ligand binding. The physiological activities of nuclear receptors differ markedly among various tissues, and depend on which co-activator binds to the nuclear receptor. Recently, focusing on differences among binding co-activators, the selective PPAR modulator (SPPARM) concept has attracted attention. The aim of SPPARM is to screen for ligands that recruit selective transcriptional cofactors such as co-activators and co-repressors of PPARs, and to express only a desired action for the treatment of disease. For example, it is expected that PPAR agonists that improve insulin resistance without causing edema or weight gain will be developed in the future, but methods based on SPPARM are not yet available.

In this study, we developed a novel ligand assay using the four-base codon fluorescence-probing method, in which a fluorescence-probed amino acid is inserted into a chosen position of a protein without denaturation,8–11) in screening PPARγ ligands. In the assay developed, sterol receptor co-activator-1 (SRC-1), which plays a critical role in insulin sensitivity and diabetes by modulating for PPARγ activity,12) was used as a fluorescence-probed co-activator. This novel assay is good in three-dimensional obstacles in protein interactions and maintaining high protein stability. Here, we propose a novel high-sensitivity, high-throughput system for screening PPARγ ligands using the assay developed.

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Abbreviations: PPAR, peroxisome proliferators-activated receptor; SRC-1, steroid receptor co-activator-1; GST, glutathione-S-transferase; RXR, retinoid-X-receptor; Tro, troglitazone; Pio, pioglitazone; 15d-ΔPGJ2, 15-deoxy-12,14-prostaglandin J2
Materials and Methods

Materials. Bixin was purchased from Nacalai Tesque (Kyoto, Japan). 15d-ΔPGJ, and farnesol were from Wako Pure Chemicals (Osaka, Japan). All the other chemicals were purchased from Sigma (St. Louis, MO) or Nacalai Tesque, and were guaranteed to be of reagent grade or tissue culture grade.

Preparation of fluorescence-probed SRC-1 proteins. The cDNA of SRC-1 corresponding to a PPAR-binding region (623–770, a total of 148 amino acids) was cloned from a human brain cDNA library by a PCR technique in an expression vector, pROX-FL92 (Protein Express, Chiba, Japan) using NcoI and Smal, in which an inserted fragment is fused to the Flag tag and His tag at the N- and the C-terminal, respectively. The sequence of CloveDirect TAMRA (Protein Express) was inserted in the Flag tag, by which TAMRA-aminophenylalanine is introduced at the ninth amino acid of the Flag tag. The final structure of the fusion protein is shown in Fig. 1A. The TAMRA-probed SRC-1 protein was expressed using pROX-FL92-SRC-1 with CloveDirect TAMRA using the RST100 E. coli HY system, and was purified using a HiTrap-HP column (GE Healthcare, Buckinghamshire, UK). Human PPARγ was expressed as a fusion protein with GST in E. coli, and was purified using a GSTrap HP column (GE Biosciences Healthcare). A fluorescence image scanner (FMBIO-III, Hitachi Software) was used to visualize the purified TAMURA-SRC-S.

Fluorescence binding assay. Fluorescence anisotropy assay was performed using a single-molecule detection system, MF20 (Olympus, Tokyo). Excitation and emission wavelengths were set at 555 and 580 nm, respectively. All slit widths were maintained at 8 nm. The cuvette chamber was equipped with a magnetic stirrer, and it had both temperature control and dehumidifying capabilities. The basic phenomenon, which allows fluorescence polarization measurement to be used to assess nuclear receptor binding, is the increase in fluorescence polarization of fluorescence-probed SRC-1 as it acquires the ability to bind to a high-molecular-weight molecule. A theoretically maximum increase in fluorescence polarization is expected because fluorescence-probed SRC-1 (approximate molecular weight, 18,000 Da) binds to PPARγ (molecular weight, 83,000 Da) in the present system.

The assessment of fluorescence-probed SRC-1 binding was made directly in terms of the measured polarization. For quantitative analysis of fluorescence-probed SRC-1 binding, an equation was derived on the basis of the law of addition of polarization values for the fraction of added PPARγ that was bound. This approach is similar to one reported previously. The fraction bound (\(B\)) is given by

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B = \frac{PM - PF}{PF - PB}
\]

where \(PM\) is the measured polarization for a given fluorescence-probed SRC-1/PPAR mixture, \(PF\) is the measured polarization of fluorescence-probed SRC-1 in the absence of PPAR, and \(PB\) is the polarization of fluorescence-probed SRC-1 when completely bound to PPAR. \(PB\) was assessed by extrapolation to an infinite PPAR concentration (derived from polarization data as a function of the PPARγ concentration). The validity of this equation depends on the assumption that there is no change in the fluorescence intensity of fluorescence-probed SRC-1-1 upon binding. Within the limits of experimental error, this assumption was verified in the present study. The amount of SRC-1 bound was assessed from the product of the total amount of fluorescence-probed SRC-1 added and the fraction of PPARγ bound. The buffer solution for assays was 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 10% v/v glycerol. A mixture of 250 nm GST-PPARγ and 5 nM fluorescence-probed SRC-1 (TAMRA-SRC-1) was added to the well. Composite or endogenous ligands (1–100 μM) were added to the GST-PPARγ and SRC-1 mixture. After a 1-h reaction, fluorescence polarization was monitored. All ligands were present at concentrations above their respective Kd values for binding to PPARγ, and also at sufficient concentrations for binding to SRC-1. In all cases, for each titration point, anisotropy was measured successively until the fluorescence polarization stabilized. The reported values are the average of five measurements after stabilization. By Scatchard analysis, the dissociation constants of the PPARγ-ligand complex and SRC-1 were estimated.

Results

Preparation of pin-point fluorescence-labeled SRC-5
Pin-point fluorescence-labeled SRC-S proteins were produced and purified using the RST100 E. coli HY system, as described above in “Materials and Methods.” After purification using a GSTrap HP column, the fluorescent-labeled SRC-S protein, which is designated TAMARA-SRC-S, was analyzed by SDS–PAGE (Fig. 1B). The fluorescent signal of the TAMRA-SRC-S was detected, corresponding to a CBB-stained protein band (Fig. 1C).

Kd values of various ligands in the binding of SRC-1 to PPARγ
Next, the effects of various ligands on fluorescence polarization were examined. As PPAR ligands, we used well-known synthesized and endogenous ones: Tro, pioglitazone (Pio), and 15-deoxy-Δ12,14-prostaglandin
Kd values were 2.89 and 21.1 μM for farnesol and bixin, respectively, although each signal was weaker than that of Pio. Scatchard plot analysis revealed that the addition of farnesol and bixin increased the fluorescence polarization change in a dose-dependent manner. Scatchard plot analysis of the binding of a PPARγ-ligand complex to TAMRA-SRC-S was estimated as follows: 83.0 nM for Tro, 221 nM for Pio, and 156 nM for 15d-ΔPGJ2 (open squares). As a control experiment, the TAMRA-SRC-S (5 nM) was incubated with GST protein (250 nM) and Tro, as indicated by open squares. After a 1-h reaction, the fluorescence polarization was measured for 10 s. The data are representative of five independent blots. All the values are means ± SEM for three tests. B, Scatchard plot analysis of the binding of a PPARγ-ligand complex to TAMRA-SRC-S in the presence (open circles) and absence (open triangles) of 385 nM Tro. As a control experiment, TAMRA-SRC-S (5 nM) was incubated with GST protein (250 nM) and Tro, as indicated by open squares. After a 1-h reaction, the fluorescence polarization was measured for 10 s. The data are representative of five independent blots. All the values are means ± SEM for three tests. D, Scatchard plot analysis of the binding of a PPARγ-ligand complex to TAMRA-SRC-S. Plots of Tro (open triangles), Pio (open circles), and 15d-ΔPGJ2 (open squares) are shown.

Evaluation of food-derived compounds by measurement of fluorescence polarization

To evaluate this novel method as a system for PPARγ ligand screening, natural compounds were examined by it. As a positive control, Pio was used. The fluorescence polarization was measured at various concentrations of PPARγ ligands (200 to 40,000 nM) in the presence of PPARγ and of SRC-1. As shown in Fig. 3A, the addition of farnesol and bixin increased the fluorescence polarization. Scatchard plot analysis revealed that the Kd values were 2.89 and 21.1 μM for farnesol and of bixin, respectively, although each signal was weaker than that of Pio.

Discussion

Improvements in fluorescent probe and photodetection techniques are becoming more and more important in biochemical studies. This is because fluorescent signals are easily detected and multisignals with different excitation/emission wavelengths are available. However, the sizes of recently used fluorescent molecules, such as green fluorescent protein (GFP), are large. Therefore, fluorescent molecules can inhibit intermolecular bindings such as protein-protein and protein-DNA binding. Moreover, even in the case of small fluorescent molecules, it is difficult to introduce them into the desired positions. In this context, the four-base codon fluorescence-probing method meets both requirements: low molecular weight (700 Da) and ease of pinpoint probing. Thus, the method is valuable for detecting molecular interactions. Ligand-dependent interactions between PPARγ and co-activators are indispensable for PPARγ-induced mRNA transcription. Indeed, adipocyte differentiation, which requires PPARγ activity, is suppressed by the ribozyme-mediated knockdown of the cAMP-response element-binding protein (CREB)-binding protein (CBP) or p300, the main co-activator for PPARγ, as previously described. Moreover, PPARγ target genes are involved in the development of diabetes and hyperlipidemia. Hence, it is important to develop a method of examining the interaction between PPARγ and its ligands.
co-activators. Co-activator pull-down assay is a valuable method for examining such an interaction in vitro, but this pull-down assay has problems, such as difficulty in quantification and low detection sensitivity. Here we report a novel quantitative method of examining the co-activator recruitment activity of PPARγ ligands. This method is highly sensitive and easy to use in quantifying the interaction. The Kd values calculated using the interaction between PPARγ and SRC-1 found in the present study are lower than those in previous reports. For example, it has been reported that the Kd values of Tro and 15d-ΔPGJ₂ are 300 nm and 11 μM, respectively.²⁰ The differences might be due to cellular processes such as the uptake and degradation of ligand compounds. The novel method is an in vitro assay that entails no cellular processes, whereas cell-based assays, such as reporter assay, are dependent on such processes. Therefore, the difference in Kd values could lead to speculations about the cellular processes specific to each ligand.

Here we found that farnesol and bixin, components of natural plants, induce interaction of PPARγ and its co-activator, SRC-1. Previously, we found that farnesol and bixin activate PPARγ in luciferase assay.²⁷,²¹ The addition of farnesol or bixin induces mRNA expression of several PPARγ target genes in 3T3-L1 adipocytes as an agonist of PPARγ. The relative Kd values of Tro (83.0 nm), farnesol (2.89 μM), and bixin (21.1 μM) are similar to the ED50s obtained in luciferase assay.²⁷,²² Therefore, it is assumed that this method is valuable for examining the moderate effects of natural compounds on PPARγ activation. In addition, it enables high-throughput screening of unknown PPARγ ligands using micro plates. Other assay systems require operations that take a longer time, such as protein fixation and cell culture.³,⁴,¹⁸,²² In the novel method, it takes approximately 2 h for the reaction to finish in a mixture of PPARγ, SRC-1, and a ligand candidate. Hence, it is expected that the processing time is shortened in comparison with other assay systems. These advantages of our method are indispensable for high-throughput screening of food-derived compounds serving as PPARγ ligands with moderate effects.

In conclusion, a novel method of examining the interaction between PPARγ and fluorescence-labeled SRC-1 is useful in the screening PPARγ ligands due to its high sensitivity and ease of operation. In addition, it may contribute to the elucidation of the various effects of co-activators with respect to the SPPARM concept, which may result in the development of novel therapies for diabetes and obesity.

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References


Fig. 3. Evaluation of Food-Derived Compounds as PPARγ Ligands by the Novel Method. A, Dose-dependent changes in fluorescence polarization using food-derived compounds, TAMRA-SRC-S (5 nm) was incubated with GST-human PPARγ (250 nm) and each natural ligand (1–40,000 nm). Tro (open circles), farnesol (open triangles), and bixin (closed circles) were used as ligands. After a 1-h reaction, the fluorescence polarization was measured for 10 s. The data are representative of five independent blots. All the values are ± SEM for three tests. B, Scatchard plot analysis of the binding of a PPARγ-ligand complex to TAMRA-SRC-S. Plots of Tro (open circles), farnesol (open squares), and bixin (open triangles) are shown.