



Engineering and optimization of an allosteric biosensor protein for peroxisome proliferator-activated receptor γ ligands

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ABSTRACT

The peroxisome proliferator-activated receptor gamma (PPAR γ or PPARG) belongs to the nuclear receptor superfamily, and is a potential drug target for a variety of diseases. In this work, we constructed a series of bacterial biosensors for the identification of functional PPAR γ ligands. These sensors entail modified *Escherichia coli* cells carrying a four-domain fusion protein, comprised of the PPAR γ ligand binding domain (LBD), an engineered mini-intein domain, the *E. coli* maltose binding protein (MBD), and a thymidylate synthase (TS) reporter enzyme. *E. coli* cells expressing this protein exhibit hormone ligand-dependent growth phenotypes. Unlike our published estrogen (ER) and thyroid receptor (TR) biosensors, the canonical PPAR γ biosensor cells displayed pronounced growth in the absence of ligand. They were able to distinguish agonists and antagonists, however, even in the absence of agonist. To improve ligand sensitivity of this sensor, we attempted to engineer and optimize linker peptides flanking the PPAR γ LBD insertion point. Truncation of the original linkers led to decreased basal growth and significantly enhanced ligand sensitivity of the PPAR γ sensor, while substitution of the native linkers with optimized G₄S (Gly-Gly-Gly-Gly-Ser) linkers further increased the sensitivity. Our studies demonstrate that the properties of linkers, especially the C-terminal linker, greatly influence the efficiency and fidelity of the allosteric signal induced by ligand binding. Our work also suggests an approach to increase allosteric behavior in this multidomain sensor protein, without modification of the functional LBD.

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1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that are activated by fatty acids to regulate the expression of genes involved in lipid homeostasis (Krey et al., 1997). Three subtypes of PPARs have been identified, including PPAR α , PPAR γ and PPAR δ/β (Committee, 1999), each of which exhibit tissue-specific expression patterns (Kliwer et al., 2001). PPAR γ is highly expressed in adipose tissue and is considered critical for fat cell development and differentiation (Chawla et al., 1994; Kliwer et al., 2001). PPAR γ has been identified as the target for the antidiabetic action of glitazones (often called “insulin sensitizers”), which are derivatives of thiazolidinedione (TZD) (Kliwer et al., 2001). PPAR γ has also been implicated in dyslipidemia, hypertension and inflammation, as well as cancer (Willson et al., 2000). Although PPAR γ exhibits a

similar domain structure to other members of the nuclear hormone receptor superfamily, the PPAR γ ligand-binding domain (LBD) is distinctly different from the canonical LBD structure. In particular, the helix 12 molecular switch of this peroxisome proliferator-activated receptor is folded back toward the predicted ligand binding pocket in the apo-form (unliganded) of the LBD, resulting in a pre-active conformation that is similar to that of the holo-forms (ligand-bound) of other nuclear receptors (Uppenberg et al., 1998).

The identification of potential ligands for modulating PPAR γ calls for highly sensitive, reliable and powerful analytical tools. Traditional binding assays, based on radioactivity (Lehmann et al., 1995), protease sensitivity (Camp et al., 2000), fluorescence displacement (Adamson and Palmer, 2002), spectrophotometry (Adamson and Palmer, 2002) and surface plasmon resonance (Yu et al., 2004), generally do not give detailed information regarding the biological function of tested ligands. Inspired by the mechanism of co-activator recruitment upon agonist ligand binding (Willson et al., 2000), transactivation assays have also been developed to monitor the biological function of ligands. Early transactivation assays relied on a cloned, full-length PPAR receptor and a PPAR response element driving the expression of a convenient reporter enzyme in a non-native host cell (Kliwer et al., 1992; McDonnell

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et al., 1993). To eliminate the effect of endogenous receptors in these host cells, chimeric receptors typically containing GAL-4 DNA binding domain (DBD) and PPAR LBD have also been constructed (Chen et al., 2004; Forman et al., 1995; Kliewer et al., 1995; Lehmann et al., 1995; Taniguchi and Mizukami, 2002). Purified coactivators can also be used for *in vitro* identification of functional PPAR ligands (Krey et al., 1997), and the binding motif of the coactivator has been used to construct a fluorescent sensor in combination with the PPAR γ LBD (Awais et al., 2007).

Recently, synthetic allosteric proteins have been engineered by several investigators via domain insertion (Ostermeier, 2005) or sequence overlap (Sallee et al., 2007) to detect the presence of ligands and modulate the function of various reporter enzymes. In general, the functional domains of artificial chimeric proteins are connected using peptides of various lengths and sequences, called linkers or spacers (Nixon et al., 1997). Studies suggest that these linkers participate in the communication between the included functional domains (Gokhale and Khosla, 2000), and that their length and sequence may affect the function and stability of the fusion protein (Gokhale and Khosla, 2000; Raag and Whitlow, 1995; Robinson and Sauer, 1998; van Leeuwen et al., 1997). Using these principles, bacterial biosensors have been recently created in our laboratories, which involve an appropriate *Escherichia coli* (*E. coli*) strain expressing an allosteric four-domain fusion reporter protein (Skretas and Wood, 2005a). In this system, LBDs from nuclear hormone receptors are inserted into an engineered intein splicing domain, which is in turn linked to a thymidylate synthase (TS) reporter enzyme. We have hypothesized that the intein domain serves as a general stabilizing scaffold, allowing the rapid development of new biosensors through simple LBD swapping with the intein domain. Several human biosensors have been generated using this scaffold, including estrogen receptors (ER α and ER β) and thyroid hormone receptor β (TR β) (Skretas and Wood, 2005a,b). Importantly, these studies suggest that the segments connecting the intein domain to the LBD play a critical role in the sensor behavior (Skretas et al., 2007; Skretas and Wood, 2005a,b).

In the present work, we created an allosteric PPAR γ ligand biosensor protein based on the modular design of our previous ER β sensor. In order to modulate the PPAR γ biosensor ligand sensitivity, the spacer peptides connecting the intein splicing domain and inserted LBD were subjected to progressive truncation. Furthermore, artificial linkers comprised of glycine and serine repeats (Huston et al., 1988) were used to replace the original ER β biosensor protein spacers in an attempt to enhance the performance of the sensor. The resulting biosensors were evaluated for growth phenotype in the presence of known PPAR γ ligands at different incubation temperatures. Finally, the mechanism of our bacterial biosensor response to ligands is proposed.

2. Experimental procedures

2.1. Reagents

Rosiglitazone (ROSIG; PPAR γ agonist) was obtained from Cayman Chemical Co. (Ann Arbor, MI), Pioglitazone (PIOG; PPAR γ agonist) from AvaChem Scientific LLC (San Antonio, TX), 17 β -estradiol (E $_2$; ER agonist), GW9662 (PPAR γ antagonist) and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$ (15D-PGJ $_2$; PPAR γ selective agonist) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Construction of the initial PPAR γ bacterial sensor protein

To generate insertional LBD fusions (Figure 1) with the *Mycobacterium tuberculosis* (*Mtu*) *recA* mini-intein (Derbyshire et al., 1997), gene splicing by overlap extension (SOE) was used

with some modifications (Horton et al., 1989). All PCR reactions described in this work were performed using Phusion DNA polymerase (Finnzymes). The DNA sequence encoding residues 232–505 of the human PPAR γ was amplified from a mammalian gene collection (MGC) cDNA clone (Accession, BC006811; Clone ID, 3447380) using forward primer Delt.Int110.PPAR γ .for and reverse primer Delt.Int383.PPAR γ .rev (Supplementary Fig. 1a). The sequences of these two primers and the ones mentioned below are shown in Supplementary Table 1. At the same time, the *Mtu* intein DNA was amplified from pMIT::ER β^* with the primers Int.Age.I.for/Int.110.rev, coding for N-terminal splicing domain of intein; and Int.383.for/Int.Xho.I.rev2, coding for C-terminal splicing domain, by PCR with overlap primers, respectively. An assembly PCR was then performed using the amplified PPAR γ DNA and two intein DNA segments using Int.Age.I.for and Int.Xho.I.rev2 as the outer primer, and the assembled DNA was used to replace the corresponding intein–LBD fusion section of pMIT::ER β^* (Skretas et al., 2007; Skretas and Wood, 2005b) by ligation into the Age I/Xho I digested vector backbone. The resulting construct, referred to as pMIT:110PPAR γ 383 (denoting the insertion of PPAR γ between residues 110 and 383 of the full-length *Mtu* intein), was transformed into calcium competent *E. coli* DH5 α for screening and amplification, and confirmed by sequencing. Confirmed clones were then transformed into the TS-deficient *E. coli* strain D1210 Δ thyA::Kan^R [F- Δ (*gpt-proA*)62 *leuB6* *supE44* *ara-14* *galK2* *lacY1* Δ (*mcrC-mrr*) *rpsL20* (*Str^r*) *xyl-5* *mtl-1* *recA13* *lacIq*] for the biosensor assay. For convenience, “pMIT:” has been omitted from the construct names throughout the text and figures.

2.3. Construction of sensors with truncated LBD-intein spacers

The PPAR γ gene was amplified from 110PPAR γ 383 using PPAR γ .for/Int.383.rev, annealing to intein sequence as illustrated above) and Int.110.for/PPAR γ .rev (Supplementary Fig. 1b). The resulting DNAs contain the full length of PPAR γ LBD sequence with an overlap sequence annealing to the sequence coding the original N-terminal or C-terminal spacer, respectively, acting as the template for assembly PCR together with intein segment DNAs. For C-terminal spacer truncation, the reverse primers including Int388.PPAR γ .rev, Int393.PPAR γ .rev, Int398.PPAR γ .rev, and Int403.PPAR γ .rev were applied to construct 110PPAR γ 388, 110PPAR γ 393, 110PPAR γ 398, 110PPAR γ 403, respectively (Supplementary Fig. 1b, right panel). For N-terminal spacer truncation, Int105.PPAR γ .for, Int100.PPAR γ .for, Int94.PPAR γ .for were used to create 105PPAR γ 383, 100PPAR γ 383 and 94PPAR γ 383 separately (Supplementary Fig. 1b, left panel). For combined N-terminal and C-terminal truncations, one primer from each of the above groups was added. The resulting constructs were digested with Age I/Xho I and ligated into a similarly digested pMIT vector. Transformations were performed using the steps mentioned above. Schematic maps of the resulting plasmids together with those described below are shown in Fig. 1. The nomenclature of the resulting clones indicates the last residue of N-terminal linker and the first residue of C-terminal linker.

2.4. Construction of sensors with GS linkers

The cloning processes were performed as described in the above section except that the primers, Int94.PPAR γ .for and Int403.PPAR γ .rev, contained the DNA sequences encoding different repeats of Gly-Gly-Gly-Gly-Ser between the PPAR γ DNA sequence and the intein overlap DNA sequence (Supplementary Fig. 1b). For convenience, “GS” was used in the context, figures and tables to denote “G $_4$ S” except for special comments. As a control, DNA fragments encoding GS linkers of different lengths were introduced into the primers Delt.Int110.PPAR γ .for and

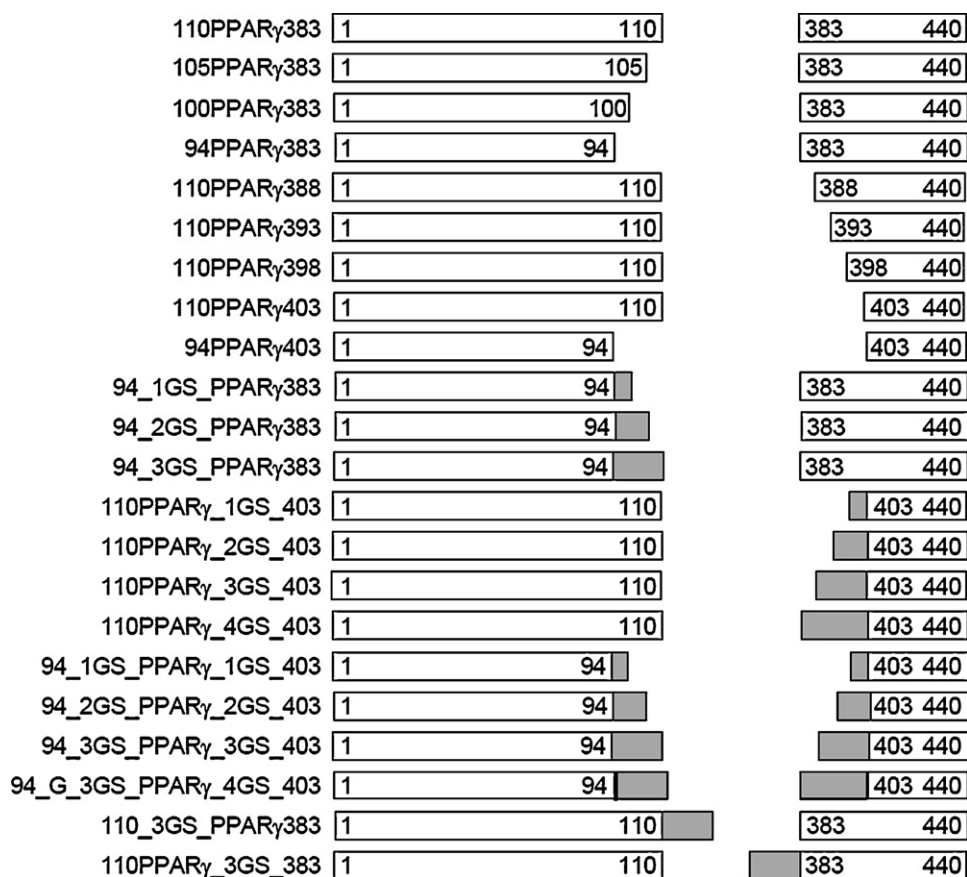


Fig. 1. Schematic diagrams of the constructed intein-PPAR γ LBD fusions with modified spacers. Open rectangles correspond to the N-terminal and C-terminal intein segments. The relative size of each intein segments is shown, with the included residues for each segment noted. Grey segments correspond to inserted GS linkers, with relative sizes shown. For the construct 94_G_3GS_PPAR γ _4GS_403, the dark line at the end of the intein indicates the insertion of an additional Glycine residue before the GS linkers. The N-terminal MBP and C-terminal TS fusions are the same for all the constructs, and are therefore not shown in the diagrams.

Delt.Int383.PPAR γ _rev, respectively. As shown in Fig. 1, the number ahead of “GS” for each construct denotes the number of “GS” unit repeats between the intein subunits and LBD at each junction.

2.5. Bacterial growth phenotypes

A single colony from a freshly streaked plate consisting of Luria Bertani (LB) agar supplemented with 100 μ g/mL ampicillin (Amp) and 50 μ g/mL thymine was used to inoculate 5 mL of liquid LB medium containing the same amount of Amp and thymine. The culture was grown at 37°C for approximately 12 h to mid-log phase, represented by an optical density at 600 nm (OD_{600}) of approximately 1.3. The culture was then diluted 1:200 into liquid thymine-free growth medium (-THY) (Belfort and Pedersen-Lane, 1984), supplemented with 100 μ g/mL ampicillin. The -THY medium used in growth phenotype tests is composed of 10 mL of 20% glucose, 10 mL of Thy Pool (2 mg/mL each of L-Arg, L-His, L-Leu, L-Met, L-Pro and L-Thr), 10 mL of 10% casamino acids solution, 200 μ L of 1% thiamine HCl, 1 mL of 0.1 M $CaCl_2$, 200 mL of 5 $\times$ Minimal Davis Broth (35 mg/mL dipotassium phosphate, 10 mg/mL monopotassium phosphate, 2.5 mg/mL sodium citrate, 0.5 mg/mL magnesium sulfate, 5 mg/mL ammonium sulfate), 4 mL of 25 mg/mL ampicillin, and q.s. to 1 L using deionized water. The glucose, Thy Pool, casamino acids and ampicillin components were sterilized by microfiltration through a 0.2 μ m membrane. All other stock solutions were sterilized by autoclaving at 121°C for 25 min, and the final medium was mixed after cooling all of the components to room temperature. All media and media stock solutions were prepared using deionized water.

To each well of a 96-well plate, 200 μ L of the diluted cell suspension was dispensed and 2 μ L of various ligands diluted into DMSO at different concentrations was applied. The resulting cell plates were shaken at different temperatures (34°C or 30°C), while the OD_{600} of each well was measured on a BioTek Synergy2 plate reader (Winooski, VT) at different time intervals. All assays were performed in triplicate, and repeated separately at least two times. The biosensor signal strength (ΔOD_{600}) is represented by the difference between the OD_{600} of the test samples and the OD_{600} of the vehicle control (DMSO only). For determinations of the half-maximal effective concentration (EC_{50}) of a given compound, the ΔOD_{600} values at a specific time where plotted as a function of the log of the ligand concentration, and fitted to a sigmoid dose–response curve using Origin software (OriginLab, Northampton, MA).

3. Results

3.1. Performance of the original PPAR γ biosensor design

Our initial bacterial biosensor for PPAR γ ligands was constructed by inserting the PPAR γ LBD between residues 110 and 383 of the *Mtu* Δ I-SM mini-intein (Fig. 1), effectively replacing the ER β LBD in our previously reported pMIT::ER β^* biosensor (Skretas et al., 2007; Skretas and Wood, 2005b). The resulting construct is referred to here as 110PPAR γ 383, and is expressed from the same backbone plasmid as our previous biosensors. At 34°C, TS-deficient cells transformed with this construct exhibited a weak TS $^+$ phenotype in the absence of ligand, as indicated by their ability to grow in -THY liquid medium (Fig. 2a). The known agonists ROSIG and

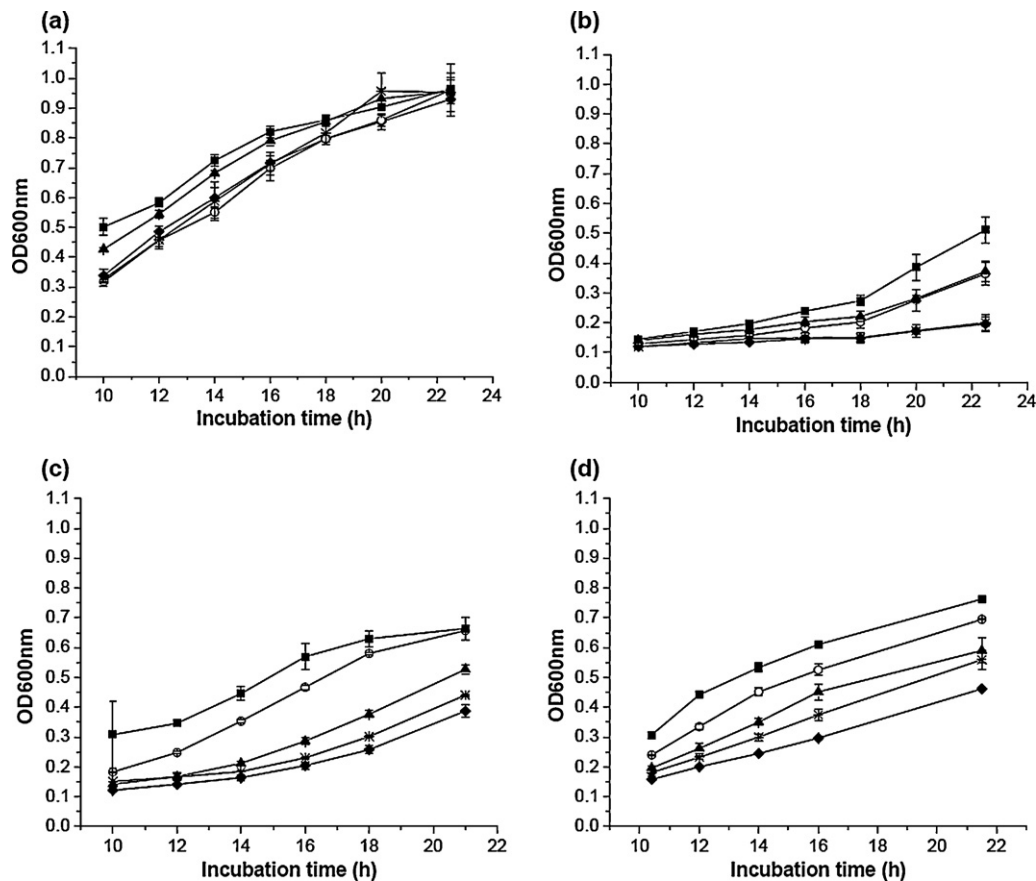


Fig. 2. Growth phenotype of representative PPAR γ biosensor strains in response to test ligands. Panels indicate growth rates for the following strains: (a) 110PPAR γ 383; (b) 110PPAR γ 393; (c) 110PPAR γ _3GS_403; (d) 94_1GS.PPAR γ _1GS.403. The strains 110PPAR γ 383, 110PPAR γ 393 and 110PPAR γ _3GS_403 were incubated at 34 °C, while 94_1GS.PPAR γ _1GS.403 was incubated at 30 °C. The final ligand concentration in each case was 10 μ M. Squares = ROSIG, triangles = PIOG, asterisks = 15D-PGJ2, open circles = GW 9662, and diamond = solvent only control (DMSO). The optical density at 600 nm (OD600 nm) is plotted as a function of incubation time. Error bars correspond to standard deviations calculated from the ODs of triplicate samples.

PIOG, however, significantly enhanced the 110PPAR γ 383 growth rate, while no significant effect was observed with the E₂ control ligand (Fig. 3). Comparatively, ROSIG caused stronger response than PIOG. A dose–response analysis of 110PPAR γ 383 (Fig. 4a and b), with ROSIG and PIOG indicated an EC₅₀ value of 3.4 ± 0.2 μ M for the former and 8.2 ± 1.1 μ M for the latter (Table 1). These results

reflect previous reports that ROSIG is a stronger agonist than PIOG, and is consistent with their reported potencies (Chen et al., 2004; Henke et al., 1998). However, the weaker native PPAR γ ligand, 15D-PGJ2 (Forman et al., 1995) did not induce a significant cell growth signal (Figs. 2 and 4c). Interestingly, the known antagonist GW9662 (Leesnitzer et al., 2002), inhibited cell growth (Figs. 3 and 4d). These

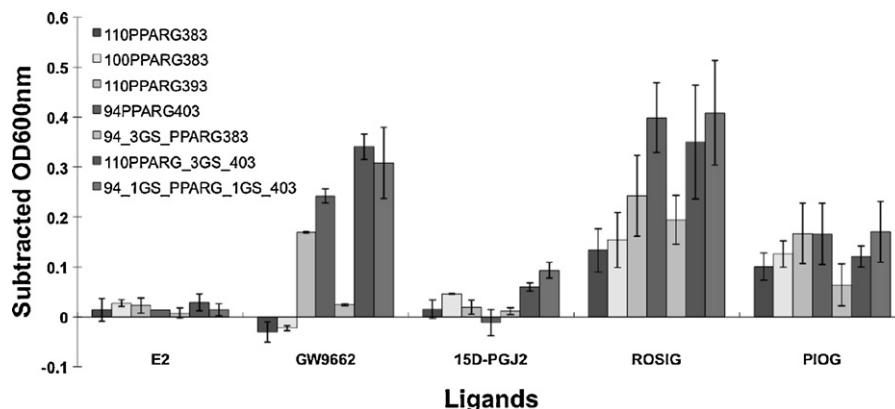


Fig. 3. Comparison of signal strength of representative PPAR γ biosensors in response to known agonist and antagonist ligands. The final concentration of each ligand is 10 μ M. The strains 110PPAR γ 383, 100PPAR γ 383, 110PPAR γ 393, 94_3GS.PPAR γ 383, and 110PPAR γ _3GS_403 were incubated at 34 °C, while 94PPAR γ 403 and 94_1GS.PPAR γ _1GS.403 were incubated at 30 °C. Optical densities at 600 nm (OD600 nm) were measured every 2 h after 10 h of incubation, and the solvent control (1% DMSO) OD600 nm measurements were subtracted from the OD600 nm measurements for cultures with test ligands. The maximum subtracted OD600 nm (subtracted OD600 nm) for E2 (non-binding control compound), GW9662 (known antagonist), 15D-PGJ2 (native ligand), ROSIG (strong agonist), and PIOG (strong agonist), within 24 h is shown here. Error bars correspond to the standard deviations calculated from two or more average values based on repetitive assays with triplicates.

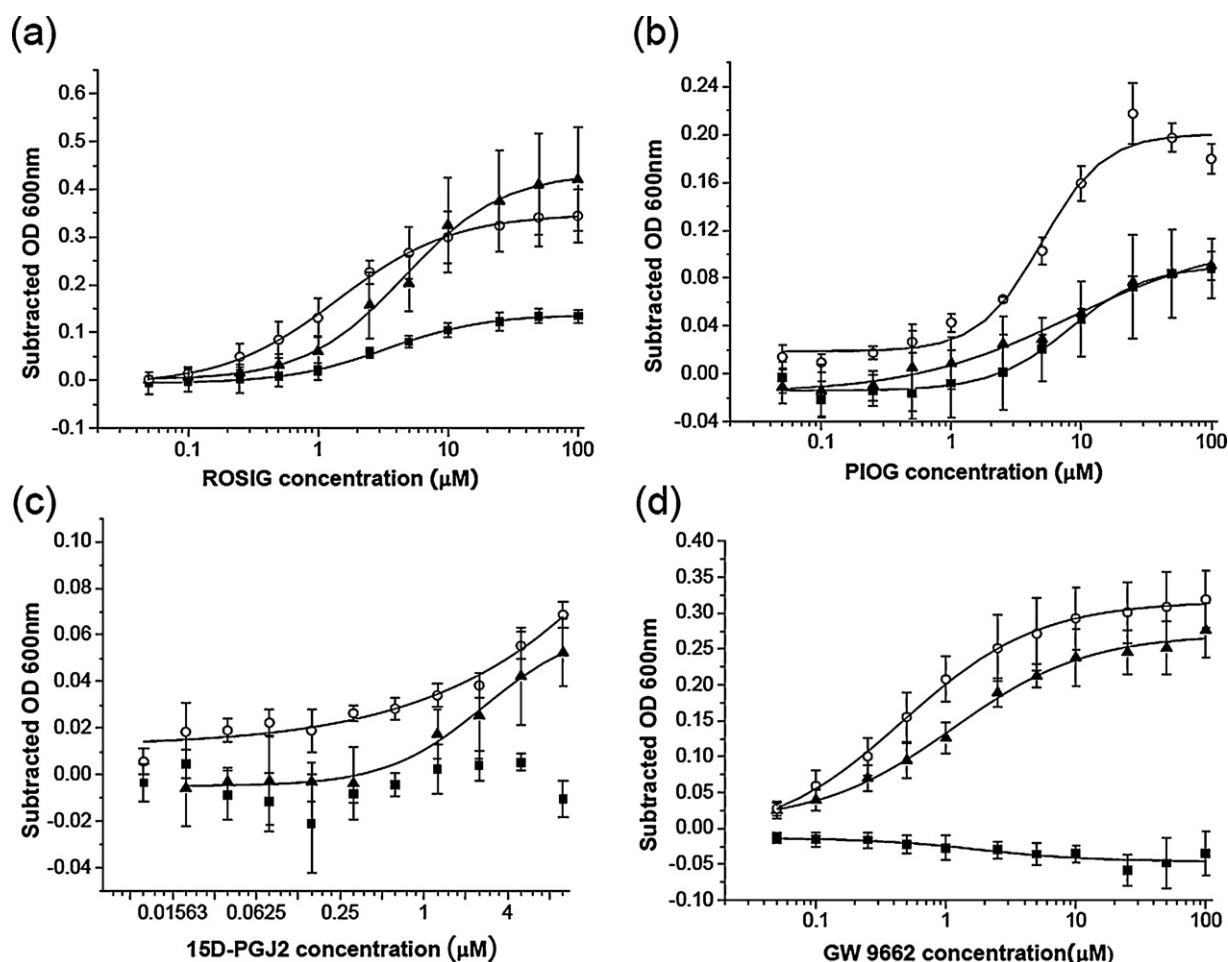


Fig. 4. Dose-dependence of ligand sensitivity of the wild type sensor and modified PPAR γ sensors. Ligands shown are (a) ROSIG, (b) PIOG, (c) 15D-PGJ2, and (d) GW 9662. Solid squares = 110PPAR γ 383, solid triangles = 110PPAR γ -3GS.403 and open circles = 94.1GS.PPAR γ -1GS.403. As in Fig. 3, after 10 h of incubation, the optical density at 600 nm (OD₆₀₀ nm) was measured every 2 h, and the maximum Subtracted OD₆₀₀ nm within 24 h total incubation was plotted against the concentration of each ligand (each curve shown was taken at a single time point). Dose-response curves were fitted to a sigmoid function for a determination of the EC₅₀ value for each compound. In panels (a) and (d), dose response curves for 110PPAR γ 383 could not be fit due to weak ligand responses. Error bars correspond to the standard deviations calculated from triplicate samples in one assay. Average EC₅₀ values based on two or more independent assays are summarized in Table 1.

observations suggest that our PPAR γ biosensor is capable of detecting and assessing potent agonist ligands, and can also distinguish agonists from antagonists, as has been observed in our previous biosensors (Skretas et al., 2007; Skretas and Wood, 2005a).

3.2. The effect of linker truncation between the intein and PPAR γ LBD

We attempted to regulate the ligand sensitivity of the PPAR γ biosensor by progressively truncating the peptide linkers connecting the intein segments to the PPAR γ LBD. As summarized in Table 2, the first group of truncations focused on the N-terminal

Table 1
Summary of EC₅₀ (or IC₅₀ for antagonism) for ligands shown in Fig. 4.

Ligands [μ M]	Sensors		
	110PPAR γ 383	110PPAR γ -3GS.403	94.1GS.PPAR γ -1GS.403
ROSIG	3.4 $\pm$ 0.2	4.6 $\pm$ 0.4	1.4 $\pm$ 0.1
PIOG	8.2 $\pm$ 1.1	8.5 $\pm$ 3.4	4.9 $\pm$ 0.8
GW9662	1.8 $\pm$ 1.2	1.1 $\pm$ 0.2	0.5 $\pm$ 0.1

Note that the EC₅₀ values shown in the table are calculated from two or more assays with triplicates, which may not be fully consistent with the observations in Fig. 4.

intein segment residues 95–110 (QPRRFDGFGDSAPIPA), while retaining the entire original C-terminal intein segment. In all cases, the PPAR γ LBD was not modified. In the absence of any ligand, the basal growth of sensor strains in this group was lower at 34 °C than that of the prototype. Notably, the 100PPAR γ 383 sensor strain, with a ten residue truncation of the N-terminal linker, displayed slightly increased sensitivity to ROSIG and PIOG at 34 °C, and was able to generate a significant response to 15D-PGJ2 as well (Fig. 3). The GW9662 antagonist exerted an inhibitory effect on the growth of the 100PPAR γ 383 strain at 34 °C as well, which was very similar to that of the prototype. Interestingly, removal of the entire N-terminal linker (94PPAR γ 383) suppressed cell growth at 34 °C regardless of ligand addition, but cell growth in the presence of ROSIG and PIOG was rescued at 30 °C. These results indicate that truncated N-terminal intein segment linkers generally decrease background growth in the absence of ligand, while improving ligand sensitivity and retaining the ability to differentiate agonist from antagonist. Large truncations, however, significantly decrease the overall activity of the sensor, as well as its ability to detect weak ligands.

Truncations from the C-terminal intein segment residues 383–402 (RVQALADALDDKFLHDMLEAE) were also examined for their effects on strain sensitivity and ability to differentiate agonist from antagonist (Table 2, group 2). The 110PPAR γ 388 strain, with a

Table 2Summary of basal growth and ligand sensitivity of different PPAR γ sensors.

Sensors	Group	Basal growth		Agonist						Antagonist	
		30 °C	34 °C	30 °C			34 °C			30 °C	34 °C
				ROSIG	PIOG	15D-PGJ2	ROSIG	PIOG	15D-PGJ2		
110PPAR γ 383	1	++++	+++	+/-	+/-	+	+	+	+/-	+/-	---
105PPAR γ 383		++	++	+/-	+	+	+	+	+/-	-	---
100PPAR γ 383		++	++	+/-	+/-	+/-	+	+	+	+/-	---
94PPAR γ 383		++	+/-	+	+	+/-	+/-	+/-	+/-	+/-	+/-
110PPAR γ 388	2	+++	++++	+/-	+	+/-	+	+	+/-	+/-	-
110PPAR γ 393		+++	+	+/-	+	+/-	++	++	+/-	+/-	++
110PPAR γ 398		++	+/-	+	+	+/-	+/-	+/-	+/-	+	++
110PPAR γ 403		++	+/-	++	+	+	+/-	+/-	+/-	+	+/-
94PPAR γ 403	3	+	+/-	++++	++	+/-	+/-	+/-	+/-	++	+/-
94.1GS.PPAR γ 383	4	++	+	+	+	+/-	++	+/-	+/-	+/-	+/-
94.2GS.PPAR γ 383		++	+	+	+	+/-	++	+	+/-	---	+/-
94.3GS.PPAR γ 383		++	+	+/-	+/-	+/-	++	+	+/-	---	+/-
110PPAR γ .1GS.403	5	++	+/-	++	+	+/-	++++	+	+/-	+	++++
110PPAR γ .2GS.403		++	+/-	+	+	+	++++	+/-	+/-	+	++++
110PPAR γ .3GS.403		++	+	+	+	+	+++	+	+	+	+++
110PPAR γ .4GS.403		++	+	+	+	+/-	+++	+	+	+	+++
94.1GS.PPAR γ .1GS.403	6	+	+/-	++++	++	++	+/-	+/-	+/-	+++	+/-
94.2GS.PPAR γ .2GS.403		+	+/-	+++	++	++	+	+/-	+/-	++	+/-
94.3GS.PPAR γ .3GS.403		+	+/-	++	+	+	++	+/-	+/-	+	+
94.3GS.PPAR γ .4GS.403		+	+/-	+	+	+	++++	+/-	+/-	+	+
110.3GS.PPAR γ 383	7	++	++	+/-	+/-	+/-	+	+	+/-	+/-	+/-
110PPAR γ .3GS.383	8	++	++	+/-	+/-	+	+	+	+/-	+/-	+

For basal growth, the data corresponds to the change in OD₆₀₀ within 12 h incubation time. Signal strengths for basal growth are defined as follows: “+/-” corresponds to an OD₆₀₀ < 0.1; “+” is an OD₆₀₀ ≥ 0.1; “++” is an OD₆₀₀ ≥ 0.2; “+++” is an OD₆₀₀ ≥ 0.3; “++++” is an OD₆₀₀ ≥ 0.4. For growth in the presence of PPAR γ ligands, the data corresponds to the subtracted OD₆₀₀ (Δ OD₆₀₀ = test compound OD₆₀₀ – vehicle control OD₆₀₀) at an incubation time where the differences in growth were maximal. Signal strengths in table for ROSIG and PIOG are defined as follows: “+/-” corresponds to Δ OD₆₀₀ ≤ 0.05; “+” is a Δ OD₆₀₀ of 0.06–0.15; “++” is a Δ OD₆₀₀ = 0.16–0.25; “+++” is a Δ OD₆₀₀ = 0.26–0.35; “++++” is a Δ OD₆₀₀ = 0.36–0.45; “+++++” is a Δ OD₆₀₀ = 0.46–0.55; “++++++” is a Δ OD₆₀₀ = 0.56–0.65. For 15D-PGJ2, signal strengths are defined as follows: “+” corresponds to Δ OD₆₀₀ = 0.04–0.08; “++” is a Δ OD₆₀₀ = 0.09–0.13. For GW 9662, signal strengths are defined as follows: “---” corresponds to Δ OD₆₀₀ ≤ -0.03; “--” is a Δ OD₆₀₀ ≤ -0.02; “-” is a Δ OD₆₀₀ ≤ -0.01; “+/-” is a Δ OD₆₀₀ = (-0.01)–0.05; “+” is a Δ OD₆₀₀ = 0.06–0.15; “++” is a Δ OD₆₀₀ = 0.16–0.25; “+++” is a Δ OD₆₀₀ = 0.26–0.35; “++++” is a Δ OD₆₀₀ = 0.36–0.45.

five-residue truncation of the C-terminal linker, displayed greater growth in the absence of ligand than the prototype at 34 °C, and no improvement in ligand sensitivity was observed. Interestingly, the 110PPAR γ 393 sensor, with a ten residue truncation, exhibited much lower growth in the absence of ligand, while ROSIG and PIOG strongly facilitated its growth and 15D-PGJ2 had almost no effect (Table 2 and Figs. 2b and 3). Surprisingly, the 110PPAR γ 393 sensor exhibited an inverted antagonistic effect with GW9662, where the addition of GW9662 increased cell growth substantially (Figs. 2b and 3). Further truncation of the C-terminal linker (110PPAR γ 398) produced responses to GW 9662 at 34 °C and to ROSIG and PIOG at 30 °C, while full deletion of C-terminal linker (110PPAR γ 403) abolished sensor growth at 34 °C irrespective of any ligand, but could be rescued by all tested agonists and antagonist at 30 °C (Table 2). Elimination of both linkers (94PPAR γ 403) produced strong positive responses to ROSIG, PIOG and GW9662 at 30 °C (Table 2, group 3 and Fig. 3). Overall, these data suggest that a truncation of ten or more residues from the C-terminal intein–LBD linker inhibits basal growth, while providing improved sensitivity to strong agonists, but an inverted antagonist activity.

3.3. The effect of GS linker insertion between intein and PPAR γ LBD

To further examine the effects of peptide linker composition on biosensor performance, we replaced the original intein linkers with different repeats of “Gly-Gly-Gly-Gly-Ser” (“G₄S”) flexible linkers (Freund et al., 1993). The first group of these sensors, included biosensor designs where the N-terminal linker was substituted with GS linkers of various lengths (94.1GS.PPAR γ 383,

94.2GS.PPAR γ 383 and 94.3GS.PPAR γ 383). These designs displayed much lower basal growth in the absence of ligands and higher sensitivity at 34 °C to ROSIG compared to their respective prototypes of identical or similar linker length in group 1 (Table 2, group 4 and Fig. 3). The antagonist GW9662 caused slight inhibitory cell growth at 30 °C (Table 2 and data not shown), but exhibited no effect at 34 °C (Table 2 and Fig. 3).

Group 5 includes sensors with GS linkers replacing the original C-terminal intein linkers (Table 2). Note that the sensors of 110PPAR γ .4GS.403, 110PPAR γ .3GS.403, 110PPAR γ .2GS.403 and 110PPAR γ .1GS.403 have the same C-terminal linker length as that of 110PPAR γ 383, 110PPAR γ 388, 110PPAR γ 393 and 110PPAR γ 398, respectively, but they exhibited much lower background growth and higher ligand sensitivity at 34 °C (Table 2). Among the designs in this group, 110PPAR γ .3GS.403 (Figs. 2c and 3) and 110PPAR γ .4GS.403 also produced promising responses to PIOG and 15D-PGJ2. Surprisingly, GW 9662 acted as a strong agonist in these sensor proteins, exhibiting a signal strength almost ten times stronger than that of the prototype. To assess the ligand potency for the 110PPAR γ .3GS.403 sensor, dose-responses were also investigated and compared to the prototype sensor (Fig. 4 and Table 1). Interestingly, while the 110PPAR γ .3GS.403 design produced a stronger signal than 110PPAR γ 383 with ROSIG and PIOG, the EC₅₀ values were similar for both (Table 1). It should be noted that EC₅₀ for 15D-PGJ2 could not be calculated due to its weak potency with these sensors. A surprising finding is that the agonist EC₅₀ for GW9662 acting on the 110PPAR γ .3GS.403 sensor is close to the antagonist IC₅₀ for this ligand on the prototype 110PPAR γ 383 sensor, despite the fact that the signals in these cases are distinctly different. These observations suggest that the C-terminal GS linker

substitution for the original linker elevates the ligand sensitivity of the PPAR γ sensors, but inverts the antagonist behavior of GW9662 without significantly changing its inherent binding properties.

The sensors included in group 6 have GS linkers replacing both of the original N-terminal and C-terminal intein linkers. In this case, basal growth in the absence of ligand was entirely blocked at 34 °C, and was significantly inhibited at 30 °C (Table 2). ROSIG was able to enhance cell growth at 34 °C, and the growth rate enhancement increased with GS linker length. Interestingly, PIOG and PGJ2 failed to have any effect with any of these linkers at 34 °C. As with the group 5 sensors, GW9662 also generated an agonist signal. At 30 °C the sensors exhibited some response to all the ligands, but unlike the behavior at 34 °C, the signal strength increased as the length of the GS linkers was reduced. In particular, the 94.1GS.PPAR γ .1GS.403 design exhibited the strongest observed response at 30 °C of any of our sensors to the weak native ligand, 15D-PGJ2 (Fig. 3). As with the previous sensors, growth–response curves were generated. In this case, the 94.1GS.PPAR γ .1GS.403 design produced a higher signal at 34 °C than that of both the prototype and the modified designs with only C-terminal GS linkers (Fig. 4). The derived EC₅₀ values for PIOG acting on 94.1GS.PPAR γ .1GS.403 are slightly lower than that of the prototype, while the EC₅₀s for ROSIG and GW9662 are much lower than that of the prototype (Table 1). These results indicate that GS linker substitutions at both LBD termini are able to not only improve the response of PPAR γ sensor to various ligands at 30 °C, but in some cases can also lead to increased apparent ligand potency.

Finally, we constructed two additional control sensors, with 3GS linkers inserted into the prototype design at either the N-terminal fusion junction (110.3GS.PPAR γ 383, group 7), or C-terminal fusion junction (110PPAR γ .3GS.383, group 8). While the former sensor exhibited behavior very much like the original prototype, the latter produced a weak positive response to GW9662 (Table 2). These data imply that in the presence of original linker, the addition of a flexible GS linker cannot efficiently improve ligand sensitivity, but can invert the signal associated with a known antagonist.

4. Discussion

In view of the clinical significance of the PPAR γ NHR, we have created reliable and economical allosteric bacterial biosensors for the identification of functional PPAR γ ligands. These sensors are not affected by endogenous eukaryotic cell factors, as with most trans-activation assays, and do not require purified NHR proteins. Further, the ligand response of the sensor can be quantified by simply measuring cell growth via a common laboratory spectrophotometer. The initially designed PPAR γ sensor exhibits a constitutive positive TS phenotype, which is distinctly different from that of our previously published ER and TR sensors (Skretas et al., 2007; Skretas and Wood, 2005a). We hypothesize that this is due to the pre-active configuration of the unbound PPAR γ LBD switch domain (apo form), which is similar to that of agonist-bound ER and TR LBDs (Uppenberg et al., 1998). This observation suggests that our sensor is able to faithfully reflect the conformational status of the apo-form PPAR γ LBD, which has not been observed in previously reported *in vitro* coactivator recruitment assays. Moreover, our sensors are able to not only assess agonist potency, but can detect intrinsic antagonists. Previous to this work, approaches to observe antagonism generally entail assays based on the competition between antagonists and agonists (Awais et al., 2007; Chen et al., 2004; Skretas et al., 2007). In this case, the signal originates from the replacement of bound agonist molecules with competing antagonists, rather than from the inherent antagonism of the antagonists. In our assay, however, GW9662 alone decreased cell growth in the absence of agonist, verifying its intrinsic antagonism. This feature can be very useful for the identification of noncompeting

antagonists. Our observations also demonstrate that modifications of the linkers connecting intein and LBD, through either truncation or GS linker substitution, are able to modulate basal growth rates of the PPAR γ biosensor strains, as well as their responses to ligands.

Previously, we proposed that the ability of our allosteric bacterial biosensor protein to correctly reflect the binding and activity of agonist and antagonist compounds is based on the transmission of a conformational change signal upon ligand binding through the intein, to the TS reporter enzyme (Skretas and Wood, 2005b). However, the sensor signal transmission process remained unclear. Based on the present observations, a hypothesis is proposed below to shed light on the mechanism of sensor signal generation, which may potentially assist the design of novel biosensors.

We hypothesize that the four domain fusion protein containing MBP, TS, the LBD and the intein splicing domain may create a crowded local microenvironment surrounding TS (Van Roey et al., 2007) (Supplementary Fig. 2), and thus sterically block TS dimerization and activation (Wood et al., 1999). The proper intein fold putatively requires the approximation of N-terminal and C-terminal intein linkers to maintain the overall intein structure (Supplementary Fig. 2). The conformational changes of the LBD inserted into the intein via these two linkers are likely to alter the orientation and distance of the intein linkers, and hence influence the intein fold. As we have previously postulated (Skretas and Wood, 2005b), TS dimerization possibly involves an intein structure perturbation. In the absence of ligand, helix 12 of a canonical nuclear hormone receptor LBD (e.g., ER α LBD) is rather mobile (Nagy and Schwabe, 2004), which would allow the intein to remain highly folded, interfere TS dimerization and cause a negative growth phenotype. Upon agonist binding, helix 12 folds over the binding site and is moved away from the LBD N-terminus (Paulmurugan and Gambhir, 2006), which would pull the C-terminal intein linker away from N-terminal linker, thus destabilizing the intein and allowing greater dimerization of TS (Supplementary Fig. 3a). In the absence of ligand, the PPAR γ sensor has a similar intein fold to that of agonist-bound ER sensor, and correspondingly exhibits significant basal growth (Supplementary Fig. 3b). In this case, agonist (e.g., 15D-PGJ2) binding only slightly increases growth, primarily by stabilizing the active form of the PPAR γ LBD (Kallenberger et al., 2003; Nettles and Greene, 2005), and this effect depends somewhat on ligand potency. In contrast, the PPAR γ antagonist moves helix 12 closer to the N-terminus, thus inhibiting TS activity and cell growth.

As helix 12 in the unbound form of the PPAR γ LBD exhibits somewhat more mobility compared to that in the bound form (Kallenberger et al., 2003), the length of the LBD-intein peptide linkers may exert significant effects on the intein fold (groups 1, 2 and 3 of Table 2). A short linker (110PPAR γ 393) will inhibit helix 12 repositioning to the active site, which allows the intein to recover a native-like fold, and thus leads to decreased basal growth (Supplementary Fig. 3c). Agonist binding is proposed to stabilize the active conformation of the PPAR γ LBD, thus changing the intein conformation and facilitating TS dimerization. As expected, increasingly potent ligands generate stronger signals. On the other hand, antagonist binding to the PPAR γ sensor with a truncated C-terminal linker will push helix 12 to a site that is away from LBD N-terminus, although not as far as the agonist site. This will be sufficient to unfold the intein and activate TS, leading to apparent agonistic behavior. In the case of a truncated N-terminal linker (group 1), helix 12 movement is probably less restricted, while the steric interference between LBD and intein may be augmented, which will also influence TS dimerization. In this case, however, the basic features associated with agonist or antagonist binding will remain.

Our results also demonstrate that the physical property of the linker may also influence the intein fold (Table 2, groups 4, 5 and 6).

Flexible GS linkers were assumed to alleviate the energy transferred to the intein by PPAR γ pre-active helix 12 configuration, allowing the intein to retain its fold and minimizing basal biosensor growth (Supplementary Fig. 3d). Benefiting from the low basal growth, the ligand-induced growth phenotype signal generated by agonist binding will stabilize helix 12 and provide extra energy to change the intein fold and activate TS. Since the C-terminal GS linker is directly connected to the position of helix 12, it is reasonable that C-terminal GS linkers (group 5, Table 2) are more favorable for the transmission of this generated energy than N-terminal (group 4, Table 2) or two-terminal GS linkers (group 6, Table 2), resulting in a higher ligand-response. The effect of GS linker length and temperature on ligand sensitivity indicates that GS linker is more flexible with increased length and temperature. It was also observed that a C-terminal GS linker exerted agonism upon antagonist binding, implying that antagonist binding to the PPAR γ LBD may also stabilize helix 12 and provide energy to drag the C-terminal GS linker away from the N-terminal linker to unfold the intein. In contrast, N-terminal GS linker retained antagonism of antagonist at 30 °C. Taken together, these observations suggest that the linkers significantly affect the intein fold, which dictates TS activity. Further, the C-terminal linker plays a critical role in distinguishing agonist and antagonists.

Table 2 also summarizes the effect of temperature on ligand sensitivity. In general, higher temperatures offer more stringent conditions for sensor cell growth, and require greater TS activity to generate a positive TS phenotype in -THY medium (Skretas and Wood, 2005a). We observed that sensor cells grew much slower at 37 °C than at 34 °C in -THY medium, and ligand sensitivity at 37 °C decreased due to a complete loss of growth at this temperature (data not shown). At room temperature, the stringency for TS activity is much lower, and the observed ligand responses became much weaker due to excessive background growth in the absence of ligand (data not shown). Therefore, we adopted either 34 °C or 30 °C for the sensor signal detection. It is apparent that all of the sensors grow faster at 30 °C than at 34 °C, and many of them exhibit improved ligand sensitivity at 30 °C. The effect of temperature on sensor behavior may be due to higher stability of the LBD at low temperature. The higher potency of ligands for 94.1GS.PPAR γ -1GS-403 at lower temperature (30 °C) than that of 110PPAR γ -3GS-403 and 110PPAR γ 383 at 34 °C (Table 1) also implies that there might be an equilibrium shift of different conformation ensemble (Gunasekaran et al., 2004; Ma et al., 1999; Vallee-Belisle et al., 2009) along with temperature change. In this case, the lower temperature would allow a more active conformation ensemble within the cells.

5. Conclusion

The modular allosteric PPAR γ bacterial biosensor design was able to detect the constitutive conformation of the PPAR γ LBD, as well as assess agonist potency and report the intrinsic antagonism of antagonists. Modifications of our PPAR γ allosteric bacterial biosensor by spacer truncation and substitution, as well as temperature optimization, produced a series of reliable tools to regulate sensor signal. This strategy would be potentially applicable to other types of biosensors based on allosteric proteins and even for the creation of artificial allosteric proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.08.006.

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