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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 (PPARγ 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 PPARγ ligands. These sensors entail modified *Escherichia coli* cells carrying a four-domain fusion protein, comprised of the PPARγ 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 PPARγ 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 PPARγ LBD insertion point. Truncation of the original linkers led to decreased basal growth and significantly enhanced ligand sensitivity of the PPARγ sensor, while substitution of the native linkers with optimized G_4S (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.

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 PPARα, PPARγ and PPARδ/β (Committee, 1999), each of which exhibit tissue-specific expression patterns (Kliewer et al., 2001). PPARγ is highly expressed in adipose tissue and is considered critical for fat cell development and differentiation (Chawla et al., 1994; Kliewer et al., 2001). PPARγ has been identified as the target for the antidiabetic action of glitazones (often called "insulin sensitizers"), which are derivatives of thiazolidinedione (TZD) (Kliewer et al., 2001). PPARγ has also been implicated in dyslipidemia, hypertension and inflammation, as well as cancer (Willson et al., 2000). Although PPARγ exhibits a similar domain structure to other members of the nuclear hormone receptor superfamily, the PPARγ 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 PPARγ 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 (Kliewer 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 PPARγ 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 (ERα and ERβ) and thyroid hormone receptor β (TRβ) (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 PPARγ ligand biosensor protein based on the modular design of our previous ERβ sensor. In order to modulate the PPARγ 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 ERβ 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 PPARγ 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; PPARγ agonist) was obtained from Cayman Chemical Co. (Ann Arbor, MI), Pioglitazone (PIOG; PPARγ agonist) from AvaChem Scientific LLC (San Antonio, TX), 17β-estradiol (E_2; ER agonist), GW9662 (PPARγ antagonist) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15D-PGJ2; PPARγ selective agonist) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Construction of the initial PPARγ 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 PPARγ was amplified from a mammalian gene collection (MGC) cDNA clone (Accession, BC006811; Clone ID, 3447380) using forward primer Delt_Int110_PPARG_for and reverse primer Delt_Int383_PPARG_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::ERβ* 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 PPARγ 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::ERβ* (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:110PPARγ383 (denoting the insertion of PPARγ between residues 110 and 383 of the full-length *Mtu* intein), was transformed into calcium competent *E. coli* DH5α for screening and amplification, and confirmed by sequencing. Confirmed clones were then transformed into the TS-deficient *E. coli* strain D1210Δ*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 PPARγ gene was amplified from 110PPARγ383 using PPARG_for/Int_383_rev, annealing to intein sequence as illustrated above) and Int_110_for/PPARG_rev (Supplementary Fig. 1b). The resulting DNAs contain the full length of PPARγ 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_PPARG_rev, Int393_PPARG_rev, Int398_PPARG_rev, and Int403_PPARG_rev were applied to construct 110PPARγ388, 110PPARγ393, 110PPARγ398, 110PPARγ403, respectively (Supplementary Fig. 1b, right panel). For N-terminal spacer truncation, Int105_PPARG_for, Int100_PPARG_for, Int94_PPARG_for were used to create 105PPARγ383, 100PPARγ383 and 94PPARγ383 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_PPARG_for and Int403_PPARG_rev, contained the DNA sequences encoding different repeats of Gly-Gly-Gly-Gly-Ser between the PPARγ 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_4S" except for special comments. As a control, DNA fragments encoding GS linkers of different lengths were introduced into the primers Delt_Int110_PPARγ_for and