Current Topics

Recent Progress in the Structural Understanding of Peroxisome Proliferator-Activated Receptor (PPAR)–Ligand Interaction

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1. INTRODUCTION

1.1. Nuclear Receptors (NRs) NRs are transcription factors that are regulated by ligands including steroids, lipids, vitamins, and cholesterol metabolites. A total of 48 NRs have been identified in humans and are involved in various physiological events such as proliferation, metabolism, homeostasis, reproduction, and inflammation, while dysfunction in NRs correlates with various diseases such as cancer, diabetes, obesity, and autoimmune disorders. Approximately 16% of U.S. Food and Drug Administration (FDA)-approved drugs are agonists or antagonists of NRs that modulate the transcription machinery and, thus, downstream gene expression.

NRs consist of several domain structures including the A/B, C, D, and E/F regions (Fig. 1A). The A/B region is an N-terminal ligand-independent activation function-1 (AF-1). The C region represents a DNA-binding domain (DBD) and has two zinc finger motifs that recognize the DNA response elements. The C region is connected to the E region via the D region, which is a flexible site with short sequences. The E/F region contains the ligand-dependent activation function-2 (AF-2) and serves as a ligand-binding domain (LBD) that not only accommodates the ligands but also associates with the coregulator proteins such as coactivators and corepressors.

1.2. Peroxisome Proliferator-Activated Receptors (PPARs) PPARs belong to the NR superfamily and are composed of three subtypes, PPARα, PPARβ/δ, and PPARγ. PPAR forms an obligate heterodimer with the retinoid X receptor (RXR), which controls the transcription of the downstream gene set. A high level of sequence and structural homology is observed among the three PPAR subtypes but they show different tissue distributions. PPARα is mainly expressed in the liver, heart, intestine, and kidney while PPARβ/δ is ubiquitously distributed to various organs but with low expression levels. PPARγ is abundantly expressed in adipose tissue, and also distributed in macrophage, kidney, spleen, skeletal muscle, and liver. PPARs play pivotal roles in lipid metabolism, inflammation, cell proliferation, differentiation, and cancer. Among the three subtypes, PPARγ is widely investigated in the fields of therapeutics and fundamental biology. PPARγ has two isoforms, PPARγ1 and PPARγ2, as the result of alternative splicing. PPARγ1 contains an additional 30 amino acids at the N-terminus compared with PPARγ2. PPARγ2 is predominantly expressed in adipose tissue while...
PPARγ exhibits ubiquitous expression.9

1.3. Molecular Function of PPARγ One main molecular function of PPARγ is the transcriptional regulation of hundreds of downstream gene sets, many of which are involved in lipid and glucose metabolism as well as inflammation.21 In particular, PPARγ is known as a master regulator of adipogenesis, which plays an indispensable role in adipocyte differentiation. These functions directly correlate with the onset of type 2 diabetes, obesity, and atherosclerosis. Generally, when PPARγ is not bound to the ligand, nuclear receptor corepressor 1 (NCoR1) associates with the PPARγ/RXR complex and suppresses the PPARγ transcription level to keep the basal level minimal.10 Once the ligand binds to the PPARγ LBD, it triggers conformational changes in the PPARγ structure, resulting in the dissociation of the corepressor complex from PPARγ. Subsequently, PPARγ forms a complex with coactivators, such as steroid receptor coactivator 1 (SRC1),15 CREB binding protein (CBP),12 mediator 1 (MEDI, also known as TRAP220/DRIP205),13 and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1alpha).14 Then, the transcription of downstream genes is activated. These processes are known as transactivation and have been characterized by many studies.

PPARγ also has other important molecular functions. Pascual et al. found that the ligand binding to PPARγ induces decoration with small ubiquitin-related modifier (SUMO), which facilitates the interaction of PPARγ with the NCoR1 complex in the promoter region of the inflammatory response genes in macrophages, and thereby represses the transcription of inflammatory genes (termed transrepression).15 In this process, PPARγ act as a suppressor for nuclear factor-κB (NF-κB). The relationship between PPARγ and NF-κB was also examined by Hou et al., who showed that PPARγ can act as an E3 ubiquitin liganse to facilitate ubiquitination of p65, a subunit of NF-κB, and its proteasomal degradation.16 In 2010, Choi et al. revealed that the Ser245 residue of the PPARγ LBD is targeted and phosphorylated by cyclin-dependent kinase 5 (Cdk5) in response to inflammation caused by obesity.71 Although the original article described this serine with residual number of isofrom 2 (Ser273), this review discusses all amino acids including Ser245 with the number of isofrom 1. The phosphorylation of Ser245 results in dysregulation of several anti-diabetic genes including adiponectin and adipokine, which can be inhibited by binding of a certain class of ligands. The binding of these ligands causes the conformational change around the Ser245 of PPARγ to be less favorable for interaction with Cdk5, which leads to transcriptional activation of antidiabetic genes and thereby creating an insulin sensitizing effect. Ohno et al. demonstrated that binding of an agonist to PPARγ induces conversion of white adipocytes into brown fat through stabilization of the PRD1-BF-1 RIZ1 homologous domain containing protein-16, followed by activation of the brown fat gene program.18

1.4. PPARγ Ligands The majority of the molecular functions of PPARγ, including those described above, are regulated by its ligand molecules, which include endogenous ligands, full agonists, partial agonists, antagonists, inverse agonists, and phosphorylation inhibitors. Endogenous ligands include the fatty acid metabolites derived from arachidonic acid, linoleic and linolenic acids,19 many of which are oxidized metabolites such as 15-deoxy-delta12,14-prostaglandin J2 (15-dPGJ2).20,21 Thiazolidinediones (TZDs) are well-known PPARγ ligands that were clinically used for the treatment of type-2 diabetes. TZDs, such as rosiglitazone and pioglitazone, act as full PPARγ agonists and induce strong transactivation of PPARγ and improve the insulin sensitivity in diabetic patients.22 However, TZDs also cause undesirable effects including body weight gain, heart failure, and edema, which often interrupted their therapeutic use. Alternatively, pharmacologists have turned their focus to selective PPARγ modulators (SPPARMs), which selectively activate the transcription of clinically beneficial genes related to insulin sensitization with less expression of unfavorable genes.24 Partial agonists show reduced transcriptional activity in a cell-based assay compared with TZDs and behave as SPPARMs in an animal model of type 2 diabetes. INT131 (also known as AMG-131)25 is one of the most potent SPPARMs and has currently progressed through phase II clinical trials.26 In the early phase of research and development of partial PPARγ agonists, an irreversible PPARγ antagonist, named GW9662, was reported by Leesnitzer et al.27 GW9662 covalently binds to the Cys285 residue through nucleophilic substitution and blocks other agonists from penetrating into the ligand-binding pocket (LBP). GW9662 along with other antagonists competitively suppress the transactivation by agonists such as TZDs and block the accumulation of lipids in adipocyte cells. Bisphenol A diglycidyl ester,28 betulinic acid,29 and G3335,30 are other known PPARγ antagonists. Lee et al. reported a high-affinity selective ligand, named T0070907, with a similar chemical structure to GW9662 but a differential effect on PPARγ transcription.31 Unlike GW9662, T0070907 suppressed the transactivation potential of PPARγ in a cell-based assay to a lower level than basal activity through facilitation of NCoR1 recruitment to PPARγ, indicating that T0070907 behaves as an inverse agonist rather than an antagonist. A recent study reported a
The development of inhibitors of phosphorylation at Ser245 of the PPARγ-LBD is also an active field of research and has been investigated in several studies. Besides rosiglitazone, non-agonist type PPARγ ligands including SR1664(33) and UHCl(34) have been identified as potent inhibitors of Ser245 phosphorylation with insulin sensitization but with less undesirable gene expression.

1.5. Overview of This Review The development of PPARγ ligands was largely supported by crystal structure analyses of the PPARγ-LBD, which in turn, prompted the design of new ligands. Additionally, the structural studies have helped to deepen our understanding of the molecular mechanism of PPARγ function. While the crystal structures provided knowledge of the ligand binding modes, there were no global differences among the structures of the apo form and agonist-bound forms of the PPARγ-LBD. It remained unclear why the PPARγ-LBD shows “graded” responses to various types of ligands, including full agonist, partial agonist, antagonist, and inverse agonist. This has led to an increased interest in understanding the dynamic conformational changes of LBDs resulting from ligand binding. This review provides an overview of a series of structural studies that report the binding modes of various types of ligands, followed by the most recent information on the dynamic mechanisms responsible for PPARγ conformational changes. These studies highlight the peculiar characteristics of the PPARγ-LBD and open the door to potential applications for drug screening and chemical labeling technologies.

2. STRUCTURE OF THE PPARγ LBD

2.1. Binding Modes of Full Agonists The conformational structure of PPARγ-LBD was firstly reported by Nolte et al.(35) They revealed the crystal structure of the apo PPARγ-LBD and the ternary complex of the PPARγ-LBD, rosiglitazone, and a nuclear receptor interaction domain of the steroid receptor co-activating factor-1 (SRC-1), which contains two LXXLL motifs. The PPARγ-LBD consists of 13 α-helices, termed H1–H12 and H-2', and a small four-stranded β-sheet (Fig. 1B). The PPARγ-LBD is positioned around helix H3, extending from helix H12 to the β-sheet lying between helices H3 and H6. The PPARγ-LBD has a large Y-shaped cavity with three subpockets where one pocket contains helices H3, H11, and H12 in the C-terminal AF-2 region. Another pocket is located around helices H2, H3, H5, and H7, while yet another pocket is surrounded by helices H2', H3, H6, and the β-sheet, which makes it a ligand-accessible site.(36,37) There is a loop structure, termed the Ω-loop, between helices H2' and H3, which is the mobile region.

Rosiglitazone binds in the LBP in a U-shaped conformation, wrapping around helix H3 with the central benzene ring of rosiglitazone directly behind helix H3 (Fig. 2). The TZD head group extends toward helix H12 to form a hydrogen bonding network with the side chains of Ser289, His323, His449, and Tyr473, resulting in the stabilization of the AF-2 region. Stabilization of the AF-2 region facilitates the association of coactivator peptides into the receptor. There are two key residues of the PPARγ-LBD involved in the interaction with the coactivator, both of which possess charged side chains and include Glu471 in the AF-2 helix and Lys301 in helix H3. These two residues are highly conserved among the nuclear receptor family, and termed “charge clamp,” that allow the orientation and placement of the helical domain HD of the LXXLL motif into the coactivator binding site. This displacement of the AF-2 region and binding manner with the coactivator are thought to be common in the nuclear receptor family because of the similarities in length, sequence, structure, and LXXLL motif binding site among the member receptors.

2.2. Partial Agonist-Bound Form The binding mode of a partial agonist in the PPARγ-LBD was initially reported by Oberfield et al.(38) GW0072 was identified as a PPARγ ligand with high affinity and weak transactivation potential. The crystal structure of a complex between the PPARγ-LBD and GW0072 was solved. In the reported structure, GW0072 occupied the region near helix H3, H7, and the β-sheet (Fig. 3A). Unlike TZDs, there was no direct interaction between the ligand and the key residues of the AF-2 regions, including Tyr473, His449, and His323. In later studies, the binding modes of various types of partial agonists were reported, many of which occupied a similar region to GW0072.40,41 The difference between full and partial agonists includes not only the binding site but also the degree of conformational changes in helix H12. This was verified in detail by Griffin’s group using a combination approach of X-ray crystallography and hydrogen/deuterium exchange (HDX) mass spectrometry analyses.25 Their HDX analysis showed that the full agonists, such as rosiglitazone, reduced the rate of amide exchange kinetics on helix H12, while this was not observed for either of the partial agonists, nTZDpa and BVT.13. Instead, these partial agonists induced robust protection from exchange in the region of the β-sheet. This was supported by the crystal structure in which hydrogen bonds between the partial agonists and some residues of β-sheet, including Ile341 and Ser342, were observed (Figs. 3B, C). At almost the same time, Pochetti et al. reported the crystal structures of complexes between the PPARγ-LBD and two synthetic enantiomeric ligands with a single chiral carbon.43 Both enantiomers exhibited similar binding modes but induced different conformational changes of PPARγ. The R-enantiomer, which behaves as a full agonist, formed a hydrogen bonding network with Tyr473,
His323, and His449, and made hydrophobic interactions with three leucine residues in helices H11 and H12. Conversely, the S-enantiomer, which partially activates PPARγ, showed fewer interactions with helix H12. These two studies suggested that a H12-independent mechanism exists to control coactivator recruitment to PPARγ in response to the binding of partial agonists.

2.3. Covalent Modification by Endogenous Ligands

The transcription of PPARγ can be activated by some natural lipids and their metabolites, including unsaturated fatty acids and oxidized fatty acids. Shiraki et al. revealed that 15-deoxy-delta12,14-prostaglandin J2 (15-dPGJ2) forms a covalent bond with the Cys285 residue in the PPARγ LBD.44) By using multiple biochemical assays, they found that lipids that possess an alpha, beta-unsaturated carbonyl moiety were attacked and conjugated with the thiol moiety of the Cys285 residue, which led to the formation of a covalent bond between the ligand and receptor. This covalent bond modification is required for the activation of PPARγ by 15-dPGJ2. The crystal structures of the complexes between endogenous fatty acids and the PPARγ LBD were firstly reported by Itoh et al.45) They clarified the binding modes of several oxidized fatty acids including oxo-lipids. 4-Oxodocosahexanoic acid (4-oxo-DHA) and 6-oxooctadecatrienoic acid (6-oxo-OTE) covalently bound to the sulfide atom of the Cys285 residue and exhibited binding poses where they appear to surround the helix H3 axis (Fig. 4A). Additionally, the carboxylates of two lipids were located in the space consisting of the polar amino acids in the AF-2 region, where they formed hydrogen bonds with Tyr473, His449, Tyr327, and His323. Thermal denaturation studies and cell-based transcription assays demonstrated that the binding of these covalent ligands stabilized the conformational structures of the PPARγ LBD with greater efficiency than rosiglitazone, resulting in activation of PPARγ transcription. This report also highlighted another important feature of the PPARγ LBP, which can accommodate multiple ligands in the LBP. This will be discussed in a later section of this review. At almost the same time, another group solved the crystal structures of the PPARγ LBD covalently bound to several endogenous fatty acids. Waku et al. found that 15-dPGJ2 exhibited a similar binding pose to 4-oxo-DHA and 6-oxo-OTE, which included covalent attachment with Cys285 and hydrogen bonding with Tyr473 and His449 46) (Fig. 4B). Notably, binding of 15-dPGJ2 drastically changed the conformation around the loop region between helix H2 and H3 and altered the position of the side chains in the vicinity of Phe287 on the PPARγ LBD surface through covalent bond formation with Cys285, which may explain why covalent ligands stabilize the PPARγ LBD. The process of conformational change of PPARγ through covalent modification was also verified by a comparison study using a crystal structure of 15-dPGJ2 and the PPARγ LBD in which Cys285 was mutated to Ser and 15-dPGJ2 was deemed to be non-covalently bound to the PPARγ LBD.47) In the non-covalently bound state, 15dPGJ2 occupied the original binding site and altered the conformation around the Ω-loop but did not affect the position of Phe287. This inactive intermediate was termed a “docked state.” Conversely, covalent modification of Cys285 modulated the rearrangement of the side-chain network around Phe287, resulting in the active conformation, termed a “locked state.” The study also revealed the bind-
ing modes of two oxo-fatty acids with different positions of the reactive group. 8-Oxo-eicosatetraenoic acid (8-oxo-ETE) and 15-oxo-ETE covalently reacted with Cys285 of the PPARγ LBD but showed different conformational changes of the PPARγ LBD. The configuration of 15-oxo-ETE, which activates PPARγ transcription, was parallel with the helix H3 axis and interacted with helix H5 and the β-sheet, while 8-oxo-ETE, which does not cause transcriptional activation, was positioned orthogonal to helix H3 and formed a hydrogen bond between its carboxylate and Tyr473. This resulted in the difference in the conformational change of the loop region between helices H2 and H3 (Fig. 4C). A recent study demonstrated that oxo-fatty acids show different reactivities to Cys285. The carboxylate of these fatty acids are commonly fixed to the AF-2 region through hydrogen bonds with Tyr473, His323, and His449, while the mobility of the reactive site is different among the fatty acids because of the flexibility of the reaction center. 6-Oxo-tetracosahexaenoic acid has a longer flexible methylene chain between the carboxylate and the reactive site compared with other oxo-fatty acids such as 4-oxo-DHA, which makes it easier to access the Cys285 because of the close proximity between reactive groups.

2.4. Phosphorylation of Ser245 One important post-translational modification of PPARγ other than covalent linkage of endogenous lipids with the Cys285 residue is phosphorylation of Ser245 (Ser273 in PPARγ2) by cyclin-dependent kinase 5 (Cdk5). Choi et al. revealed that this phosphorylation correlates with insulin sensitivity that can be altered by obesity and dysregulates the expression of a subset of genes including anti-diabetic genes. The phosphorylation of Ser245 in the PPARγ LBD by Cdk5 can be downregulated by the binding of several ligands, such as rosiglitazone, MRL24, and non-agonist type ligand (UHC1). Crystallographic and HDX analysis showed that these ligands stabilized the region near the β-sheet and helix H3, which froze the phosphorylation site in a configuration less favorable to Cdk5 enzymatic activity. A recent study demonstrated that the stabilization around these regions can occur by either direct or indirect mechanisms. MRL24, known as a partial agonist, inhibits Ser245 phosphorylation through direct binding to the site near the β-sheet and helix H3, which is termed the alternate site and is important for conformational changes of Ser245. The S-enantiomer of LT175 mainly occupies the AF-2 region far from the alternate site but stabilizes the β-sheet and helix H3, termed “allosteric blocking” of PPARγ phosphorylation. This long-distance inhibition mechanism is explained by sequential alternation of the conformation from helix H3 to the phosphorylation site. In the case of LT-175, the orientation of the phenyl ring in Phe282 is shifted, allowing it to form van der Waals interactions with the Leu356 side chain. This in turn triggers the hydrophobic interactions among Leu356, Leu353, Ile281, Met348, Ile249, and Leu255, and suggests that there is cross talk of hydrophobic interactions occurring among these residues.

2.5. Dynamic Mechanism of Ligand Binding The crystal structures of PPARγ-ligand complexes provide us information of the specific ligand binding mode in the receptor protein. Conversely, the backbone folds of the protein structure were relatively similar among the studies using various types of ligands, including full agonists, partial agonists, and the apo form. This suggested that the graded agonism of these ligands were regulated by receptor dynamics. The dynamics of the conformational change of the PPARγ LBD was firstly analyzed by Johnson et al. using NMR spectroscopy. The three-dimensional NMR experiments of the apo PPARγ LBD showed less cross-peaks than expected, while the missing peaks were restored on the binding of rosiglitazone. Berger et al. demonstrated that the restoration of the missing peaks by TZDpa, which selectively modulates the transcription of the PPARγ target genes, was partial when compared with the full agonist. These results suggested that the conformation of the apo PPARγ LBD is in a highly mobile state and can be stabilized by the binding of ligands with graded restoration corresponding to the degree of transcriptional outputs. This was further verified by Kojetin’s group. They compared the changes of NMR signals among the apo PPARγ LBD and LBDs associated with several types of ligands, such as full, partial, and intermediate ligands, and reconfirmed the previous observations of graded restorations. They also explained that the absence of several peaks of residues in the apo form was likely because of the time scale of the NMR resonances. Generally, NMR is sensitive to the structure and dynamics of proteins on time scales ranging from pico-seconds to second, and the slower dynamics gives peaks with multiple populations. In their analyses, the partial agonists MRL24 and MRL20 displayed multiple peaks of several residues in helices H3, H4, H5, H8, H10, H11, and H12 with broad resonances, termed slow conformational exchanges on a time scale of milliseconds or slower. Conversely, single peaks of the same residues were observed in the case of a full agonist, suggesting that the ligand-induced stabilization correlates with the graded transcriptional response of the ligand. This was supported by a recent investigation using fluorine-19 (19F) NMR and molecular simulations. Chrisman et al. examined the structural changes of the co-regulator binding surface in the PPARγ LBD, including helix H12, and demonstrated that partial agonist-bound PPARγ, as well as the apo form, exhibited multiple thermodynamically accessible conformations, while full and inverse agonist binding was consolidated into a few conformations that favored the recruitment of a coactivator or corepressor, respectively.

2.6. Repressive Mechanism and Transition to the Active State Recent structural studies have put more focus on the repressive mechanism of PPARγ transcription by inverse agonists. Compared with full and partial agonists, the structural mechanism of the repression state is not fully understood. Brust et al. analyzed the conformational changes of the PPARγ LBD bound to T0070907 and GW9662, an inverse agonist and a transcriptionally neutral antagonist, respectively. Both compounds possess the same chemical scaffold but contain subtle differences where the phenyl ring of GW9662 is replaced with a nitrogen-containing pyridine ring in T0070907. They covalently attached to the Cys285 residue in helix H3 but displayed different effects on transcription; T0070907 suppressed PPARγ transcription activity while GW9662 showed negligible effects on the basal level. The researchers solved the crystal structure of the T0070907-bound PPARγ LBD (Fig. 5A) in which the pyridyl group of T0070907 formed a water-mediated hydrogen bond network with Arg288 that is essential for the recruitment of the corepressor to the PPARγ. NMR analysis showed that T0070907-bound PPARγ exchanges between two long-lived conformations, one of which is shared...
with the GW9662-bound state, and the other is a unique state that favors corepressor binding. A recent study further verified this structural diverse mechanism of the inverse agonist-bound state by analyzing a tertiary complex of PPARγ-LBD, T0070907, and the corepressor peptide.\(^5\)\(^4\) The researchers solved the crystal structure in which the helix H12 was displaced from the solvent-exposed conformation and occupied the ligand entry site consisting of helix H3, the Ω-loop, and the β-sheet, which favored corepressor binding (Figs. 5B, C). Notably, the binding poses of T0070907 in the PPARγ LBD were different among the complexes with or without corepressor. In the crystal structure of the PPARγ LBD without corepressor peptides, the pyridyl group of T0070907 was located in the region near helix H3, H5, and the Ω-loop, while the corepressor-bound LBD accommodated the pyridyl group of T0070907 in the AF-2 surface. The researchers suggested that these two different binding modes of T0070907 may correlate with the exchange between transcriptionally active and repressive conformations of helix H12. NMR analyses also revealed that helix H12 in the apo form exchanges between transcriptionally active and repressive conformations. Bruning’s group reported a similar model of a corepressor-favored conformation based on the crystal structure of the PPARγ LBD bound to non-covalent inverse agonists such as SR10171 and SR11023.\(^5\)\(^5\) These studies proposed a refined model of ligand-induced activation of PPARγ transcription where agonist binding alters the position of helix H12 from adopting a repressive conformation around the ligand-entry site and instead stabilizes a solvent-exposed active helix H12 conformation that recruits coactivator proteins containing the LXXLL motifs (Fig. 5D). More recently, it has been reported that this type of structural change induced by ligand binding to the PPARγ LBD can occur through an induced-fit mechanism with a two-step conformational alteration including an initial fast step followed by slow conformational change.\(^3\)\(^6\)

2.7. Cobinding of Multiple Ligands One of the prominent features of the PPARγ LBP is a large cavity that consists of multiple sub-pockets termed the Y- or T-shaped cavity. Several structural analyses revealed that the PPARγ LBP can accommodate more than two ligands in the cavity simultaneously. The actual binding modes of multiple bindings were firstly reported by Itoh et al.\(^3\)\(^5\) They reported that the PPARγ LBP was occupied by two molecules of hydroxylated fatty acids, including 9-(S)-hydroxyoctadecadienoic acid (9-HODE) and 13-(S)-hydroxyoctadecadienoic acid (13-HODE), which are not covalent modifiers. The first molecule of 9-HODE interacted with the AF-2 region around helices H3, H11, and H12, while the second 9-HODE was positioned between helix H3 and the β-sheet (Fig. 6A). Based on the versatility of the LBP, the authors hypothesized that the large cavity of the PPARγ LBP is likely to have a sensor role for cellular oxidized fatty acids. Morikawa and his colleagues revealed that two distinct ligands can bind to the LBP simultaneously.\(^5\)\(^7\) In their structural model, each sub-pocket accommodates a different ligand: 5-methoxy-3-indoleacetic acid (5-MIA), a serotonin metabolite, occupied the AF-2 region while 15-oxo-ETE bound to the site around helix H3 and the β-sheet through the covalent modification of Cys285 via a Michael addition reaction in the presence of 5-MIA (Fig. 6B). Interestingly, these two ligands synergistically activated PPARγ transcription in a cell-based assay. They proposed possible roles of each sub-pocket for sensing cellular distinct metabolites to respond to the two independent metabolic pathways involving serotonin and the fatty acid.

Cobinding of multiple ligands in the LBP was also assessed by NMR analyses that revealed two molecules of synthetic ligands, MRL20 or MRL24, bound to PPARγ.\(^3\)\(^8\) The first molecule of the MRL ligand occupied the AF-2 region, which was determined to be a canonical site designated by the authors, whereas the second molecule bound to the region around helix H3, the β-sheet, and the Ω-loop. They defined the binding site of the second molecule as an alternate site. They also showed that alternate site binding is not blocked by synthetic antagonists like GW9662 or T0070907. This was also verified by biochemical assays, which revealed that addition of MRL20 or MRL24 modulated coregulator recruitment in the presence of both synthetic antagonists and thereby PPARγ transcription. Binding of MRL20 to the alternate site also occurred in PPARγ covalently bound to endogenous fatty acids including 15-dPGJ2 and 15-oxo-ETE. Cobinding of synthetic and natural ligands (lipids) was further investigated by the same group.\(^3\)\(^9\) Their biophysical experiments demonstrated that synthetic ligands, like TZDs and covalent antagonists, bind to the canonical site and push the natural ligands to an alternate binding site near the Ω-loop (Fig. 6C). Importantly, the pushed ligands can be still anchored to the loop region because of their affinity to the alternate site, which modulates PPARγ structure and function synergistically together with the synthetic ligands. A similar phenomenon was observed from the crystallographic analysis of the complex between the PPARγ LBD and luteolin, a natural flavonoid, in which myristic acid co-occupied the LBP.\(^6\)
3. APPLICATIONS

3.1. Approaches for PPARγ Ligand Design

Several structural analyses of the PPARγ LBP provide considerable information on the characteristics of the LBP and can help to guide the development of ligands. Indeed, recent studies reported several approaches for the design of new PPARγ ligands by focusing on the characteristics of the LBP. These trials enabled to not only create new structures of ligands but also facilitate the repurposing of existing molecules with chemical elaboration and/or modification, which confer distinct functions to the original compounds. Several groups noticed “incompleteness” of GW9662, an irreversible antagonist, and proposed some approaches for development of new covalent drugs by using its chemical scaffold. Our group used the large cavity of PPARγ LBP and proposed the two-step cell-based strategy for the ligand development and reported synthetic covalent agonists61) (Fig. 6D). In the first step of the strategy, the combination of ligands that simultaneously bound to the LBP and cooperatively activated transcription was identified by a conventional cell-based assay. Second, the two identified ligands were fused into a single hybrid structure. We found that a plant-derived cinnamic acid derivative activated PPARγ transcription in combination with GW9662, an irreversible antagonist. Generally, GW9662 covalently reacts with Cys285 in the center of the LBP through nucleophilic substitution, resulting in blocking the binding of other ligands such as TZDs. However, there is an additional space near helix H3, the Ω-loop, and β-sheet, which can accommodate another ligand. This space was also termed an alternate site by Kojetin’s group.58) Our docking model suggested that the cinnamic acid derivative could occupy the alternate site in the presence of GW9662 and likely stabilize helix H3, the Ω-loop, and β-sheet region. We designed and synthesized a hybrid structure of the two compounds and confirmed its potent PPARγ agonistic activity with a degree of partial transcriptional potential when compared with a full agonist through covalent binding to the Cys285 residue. For the design of a hybrid structure, the size of linker and the position of two units are important for PPARγ agonistic activity. Indeed, we confirmed this point by synthesizing several chemical derivatives in which the orientation and proximity of

Fig. 6. Characteristics of Large Cavity of PPARγ Ligand-Binding Pocket

The crystal structure of PPARγ LBD bound to two molecules of 9-HODE (PDB code: 2VSR) (A),45) the pair of 5-MIA and 15-oxo-ETE (PDB code: 3ADW) (B),57) the pair of rosiglitazone and oleic acid (PDB code: 6MD4) (C)59) are shown. (D) The schematic diagram of ligand-linking strategy.61) The pair of ligands that cooperatively activate PPARγ transcription is identified by cell-based conventional assay. Then, the hybrid structure of the two is designed and synthesized. (E) “Repurposed” structures of classical ligands and its chemical derivative. (Color figure can be accessed in the online version.)
These studies suggested that the chemical scaffold of GW9662 can be used for further decoration with an additional lipophilic moiety and/or functional groups that confer a distinct function (Fig. 6E). Kojetin’s group paid attention to the space for the alternate site in GW9662-bound PPARγ that is occupied by MRL20.63) This allosteric binding of MRL20 caused PPARγ transcription activation even in the presence of GW9662, which highlights the incompleteness of GW9662. They designed a series of derivatives of GW9662 with expanded moieties and identified SR16832 (Fig. 6E) as a dual-site covalent inhibitor that blocked the ligand from binding to both the canonical AF-2 pocket and the alternate site in the PPARγ LBP. Park and colleagues designed GW9662 derivatives with a large lipophilic group attached onto the aromatic ring of GW9662.64) The resulting compounds, including SB1453 (Fig. 6E), stabilized the sheet region and the phosphorylation site, thereby blocking the phosphorylation of Ser245 of the PPARγ by Cdk5 in an irreversible manner because of the function of the 2-chloro-5-nitrobenzoyl group of the GW9662 unit. They also developed a reversible covalent inhibitor for Ser245 phosphorylation by substituting the covalent reactive group of GW9662 with a cyano-acrylamide group,65,66) named SB1495 (Fig. 6E), which reversibly formed a covalent bond with the cysteine thiol group.67,68) Recently, Hinnah et al. developed photoswitchable PPARγ ligands in which the aminoethoxy linker of rosiglitazone or GW1929 was substituted with an azo group69) (Fig. 6E). The ligands can change their configuration under light irradiation, resulting in the activation of PPARγ transcription.

3.2. Chemical Labeling

The strategies to label a specific protein with a chemical fluorophore have emerged as useful tools for investigation of protein function and also ligand screening in vitro and in living cells. Given that proteins have many reactive functional groups, site-specific modification of proteins is indispensable for precise detection in living systems. The Cys285 residue is the reactive center of the PPARγ LBD, which can be modified by electrophile compounds and used as the target of selective labeling. Tsukidate et al. developed a strategy for chemical labeling of the PPARγ LBD by using a GW9662 derivative where the alkyne group was attached to its aromatic ring for the biorthogonal reaction (Fig. 7A). The alk-GW9662 can bind to the Cys285 through covalent modification and be labeled with azide-rhodamine B

![Fig. 7. Strategies for Chemical Labeling of PPARγ LBD](Image)
via a copper-catalyzed azide-alkyne cycloaddition reaction. In cells, the labeling of the Cys285 on the PPARγ LBD with alk-GW9662 is competitively inhibited by binding of other ligands such as TZDs, which highlights the usefulness of the tool for screening and characterization of candidate PPARγ ligands. Using the developed tool, the authors identified conjugated (9E,11E)-linoleic acid, a microbait-derived metabolite, as a partial PPARγ agonist. Kojima et al. used an unsaturated lipid that contained two reactive groups (Fig. 7A), an enone for labeling the Cys285 of PPARγ and an azide for attachment with an azadibenzo-cyclooctyne (ADIBO)-conjugated fluorophore through the Huisgen reaction.73) Although their intracellular labeling trials were not successful, they detected the labeling of the PPARγ LBD on the proteome using an in vitro cell-free experiment. The same group reported an alternative strategy for labeling of PPARγ and the vitamin D receptor (VDR) with improved sensitivity for a cell experiment using a turn-on probe.72) The turn-on probe possessed an ynoate group as a precursor of a fluorophore that reacts via the proximity effect with the nucleophilic side chain of an amino acid following the capture of the target receptor via interactions with the ligand unit (Fig. 7B). Next, subsequent lactonization between the resultant enoate and phenol group is induced by the geometric proximity effect, which leads to the formation of the coumarin fluorophore. The probe was demonstrated to label the VDR and PPARγ in both cell-free and cell-based experiments in a turn-on manner. Notably, their turn-on probe can be attached with a short peptide, such as the partial sequence of a coactivator instead of small molecule ligands and be used to label the receptor protein.

4. CONCLUSION

This review provides an overview of the structural changes of the PPARγ LBD in response to the binding of various classes of ligands. A growing body of studies on the mechanisms responsible for the conformational changes of PPARγ provides us with new knowledge of the dynamics of ligand-receptor interactions. These works inspire medicinal chemists to propose idea for repurposing of classical agonist or antagonist into a new class of PPARγ ligands, including covalent ligands, photoswitchable activator, and chemical labeling probes. The findings and methodology summarized herein may be broadly applicable to studies on the structural mechanisms of other NRs because of the similarities among the NR group. In particular, it still remains unclear how partial agonists dynamically regulate the conformational changes, how SPPARM/ partial agonists structurally select a favorable coactivator, and whether the proposed model is actually seen in living cells. Further development of new techniques and tools will address these questions and deepen our understanding of the dynamics of the PPARγ LBD, and lead to greater pharmacological control of function with ligands.

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