Unique Interactome Network Signatures for Peroxisome Proliferator-activated Receptor Gamma (PPARγ) Modulation by Functional Selective Ligands

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The nuclear receptor PPARγ regulates adipogenesis and plays a central role in lipid and glucose homeostasis, and is the molecular target of the glitazones (TZDs), therapeutics used to treat insulin resistance and type-2 diabetes (T2D). Although the TZDs, which are PPARγ agonists, demonstrated robust clinical efficacy in T2D, their use has been hampered by an array of untoward side effects. Paradoxically, partial agonists (e.g. MRL242), antagonists (e.g. SR1664), and inverse agonists (e.g. SR10171 and SR2598), possess similar insulin-sensitizing efficacy as the TZDs in obese diabetic mice. Given the unique pharmacology of these modulators, we sought to identify the components of the PPARγ transcriptional complex that is regulated by these ligands. To achieve this, we employed subcellular fractionation of adipocytes combined with either trapping of the receptor complex on biotinylated DNA oligonucleotide, or classical immunoprecipitation. Tandem mass spectrometry analysis revealed unique, partially overlapping, compound- and subcellular compartment-specific complexes. Components of these interactomes are putative coregulators of PPARγ. Interestingly, complexes isolated in the cytosol contain sets of proteins involved in cellular assembly and extracellular matrix. Furthermore, the interactome observed for cytosolic non-DNA bound receptor was distinct from that observed from nuclear chromatin associated PPARγ, suggesting cellular compartment-specific roles for this receptor. Molecular & Cellular Proteomics 16: 10.1074/mcp.RA117.000308, 2098–2110, 2017.

Peroxisome proliferator-activated receptor gamma (PPARγ; NR1C3) is a member of the nuclear receptor (NR) superfamily of ligand-modulated transcription factors. There are two distinct isoforms of the receptor, PPARγ1 and PPARγ2, that originate from the NR1C3 gene via the use of separate start sites and 5′ exon usage leading to a 28-amino acid N-terminal extension in human (30 in mouse) for the γ2 isoform. PPARγ1 is ubiquitously expressed, whereas PPARγ2 is limited to adipose tissue (1, 2) and is a master regulator of adipogenesis (2–23). NRs are multidomain proteins that consist of an intrinsically disordered N-terminal domain (A/B domain) containing a ligand-independent activation motif AF1 that can be modulated by PTMs and interaction with other transcription factors. The A/B domain connects to a highly conserved two-zinc finger DNA binding domain (DBD; C domain) followed by a flexible and sequence diverse hinge domain (D domain). The hinge attaches the structural conserved ligand-binding domain (LBD; E domain) that contains the ligand binding pocket (LBP) and a ligand-dependent activation motif called AF2. Receptor ligands bind within the hydrophobic LBP altering the hydrogen bond network of the LBD resulting in changes in the conformational plasticity of AF2 to form a binding surface facilitating docking of transcriptional coregulatory proteins to the NR. For example, agonists binding to an NR stabilize its active conformation via closure of AF2 surface allowing coactivator recruitment, whereas antagonists or inverse agonists either destabilize or reorient the AF2 surface into an inactive conformation favoring binding to transcriptional corepressor proteins.

PPARγ is the molecular target of the glitazones (thiazolidinediones or TZDs) that include rosiglitazone (RSG; Avandia) and pioglitazone (PIO; Actos) (14–16). Despite demonstrating robust and durable efficacy in type 2 diabetic patients, safety concerns over their use has grown. Although weight gain is associated with the use of TZDs, the major safety concerns

LBP, ligand binding pocket; AF1 and AF2, activation function 1 and activation function 2; TZDs, thiazolidinediones; RSG, rosiglitazone; PIO, pioglitazone; PVE, plasma volume expansion; HDX, hydrogen deuterium exchange; MS, mass spectrometry; ΔL1 cell, differentiated MEF 3T3-L1 cells; MEF, mouse embryonic fibroblast; MSCs, mesenchymal stem cells; WB, western blot; IP, immunoprecipitation; IPA, ingenuity pathway analysis; qPCR, quantitative polymerase chain reaction.
include edema, plasma volume expansion (PVE) which may be associated with cardiomegaly, increased risk of bone fractures, and specific to pioglitazone an increased risk of bladder cancer. Studies in animal models and in man have shown that indicators of weight gain and PVE, although not eliminated, can be minimized without loss of insulin sensitization efficacy using partial agonists of PPARγ (17, 18). These studies clearly demonstrate that insulin sensitization afforded by PPARγ ligands does not correlate with the magnitude of agonism of the receptor that they induce. Additional studies demonstrated that the insulin sensitization afforded by TZDs, partial agonists, antagonists, and inverse agonists in diabetic mice correlates with the ability of these ligands to decrease phosphorylation of PPARγ at S273 (pS273) and blockage of this PTM correlates with an increase in expression of a subset of target genes associated with insulin resistance (19–21). These studies suggest that agonist activation of the receptor controls proadipogenic genes, whereas pS273 levels impact expression of the anti-diabetic genes. These also support the notion that ligands can be designed that block pS273 without agonizing the receptor, and such ligands have been defined as functional selective PPARγ modulators (20). Biophysical and structural analysis integrating cocrystallography, mutagenesis, and the hydrogen/deuterium exchange (HDX) were used to reveal the structural mechanism for these pharmacologically diverse ligands (20).

With distinct structural mechanisms and unique pharmacological profiles for this set of modulators we sought to determine the impact that these compounds have on the endogenous PPARγ interactome isolated from differentiated MEF 3T3-L1 cells (∆L1 cells) treated with a set of functionally distinct ligands. However, NRs, like most transcription factors, are generally underrepresented in global proteomic studies given their relatively low abundance. To circumvent this problem, methods including overexpression of tagged-protein (Flag-tag or Myc-tag), cross-linking intervention (RIME) (22), or reconstitution of recombinant protein in cell lysates are commonly applied (23). However, these approaches could alter the physiologically relevant interactome. Recently, Foulds et al. employed an oligo trapping approach to probe the interactome of the estrogen receptor (ER) and sequence-specific DNA affinity trapping have been used before by others to study transcription factors (24, 25). As outlined in Scheme 1 and Fig. 1, we followed a similar approach to compare the endogenous PPARγ interactome within ∆L1 cells treated with a set of functionally distinct ligands by classical immunoprecipitation using a ChIP grade PPARγ specific antibody and compared these data with that obtained using a biotinylated oligo containing a 3× tandem canonical direct repeat 1 ((5′AGGTCA n AGGTCA 3′)₃ or 3xDRI) which is representative of the canonical PPARγ response element (PPRE).

The PPARγ interactome network was analyzed from two cellular environments, the cytosol where the receptor is translated and the nucleus where the receptor resides post translocation. Members of the steroid hormone subfamily of NRs (Type I NRs) such as the glucocorticoid receptor (GR) and the androgen receptor (AR) are known to reside in the cytosol associated with chaperones. The chaperone complexes assist ligand binding to these receptors which then triggers dimerization and nuclear translocation (26–29). In contrast, it is generally thought that Type II NRs such as PPARγ reside almost exclusively in the nucleus despite studies which have shown that the Type II NR VDR (vitamin D receptor) can activate signal transduction pathways on ligand engagement in the cytosol (30). These studies and others suggest non-genomic functional roles for Type II NRs. Regardless, most PPARγ proteomic studies have focused exclusively in the context of whole cell lysates or nuclear fractions. Therefore, the role of PPARγ and its interactome in the cytosolic remains poorly understood. To address this, we present a qualitative analysis of the ligand-dependent interactome of endogenous PPARγ in both the cytosolic and nuclear environment of differentiated adipocytes.

MATERIALS AND METHODS

Chemical Reagents and Antibodies—SR1664 and SR2595 compounds were synthesized in-house. RSG, MRL24, insulin, dexamethasone (DMX), isobutylmethylxanthine (IBMX), and DMSO were purchased from Sigma Aldrich (St. Louis, MO). Primary mouse monoclonal anti-human PPARγ (sc-7273; 408–505; Lot #: L3013) for IP, rabbit polyclonal anti-human PPARγ (sc-7196; 8–108; Lot#: A2512) for Western blot (WB), and mouse IgG (sc-2025; Lot#: C1915) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) Mouse monoclonal anti-human PPARγ2 (PP-K8450B-00; 1–28; Lot#: A-2) for WB was obtained from R&D Systems (Minneapolis, MN). Hsp90, Histone3, Hdac2, and Gapdh antibodies were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies goat anti-rabbit IRDye800 and goat anti-mouse IRDye680 were from LI-COR Biotechnology (Lincoln, NE). Immunoprecipitations were performed with Protein A/G conjugated magnetic beads purchased from Pierce Biotechnology (Waltham, MA).

3T3-L1 Cell Culture and Adipocyte Differentiation—The cell line was purchased from ATCC, and the differentiation protocol was based on previous publications with slight modification (31, 32). Specifically, cells were grown in 15-cm culture dishes (ten to fifteen million cells per dish) with Dulbecco’s modified Eagle’s medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Plates were incubated at 37 °C in a controlled atmosphere with 5% CO₂. After 2 days of reaching confluence, cells underwent induction by replacing the media with differentiation media containing 1 μM DMX, 0.5 mM IBMX, and 850 nM insulin. After 48 h, media was replaced with DMEM containing 10% FBS plus insulin. For 4-day and 1-day ligand treatment, the
compound was either added to cells immediately following post induction or 3 days later, respectively.

Subcellular Fractionation—Cytoplasmic and nuclear isolation were performed using a Pierce kit (PI78840). Because of high salt content (MgO, and NaCl) in the nuclear extract buffer, the total volume was diluted 1:1 with 1×PBS before carrying out the pull-down procedure which was like that used for the cytosolic pull-downs. Approximately 20–30 million cells were used per pull-down per condition.

Biotinylated Oligo—DNA duplex oligos were purchased from IDT (Corvallis, IA) with the sequence specificity of the forward 3×DR1 DNA oligo was 5'-biotin-cgcggaacctAGGTCAAAAGGCTCAA-AGGTCA AAGGTCGAAAGGCTGaattcgcg-3’. The forward Scro DNA oligo is 5'-biotin-cgcggaacctTAATACAACGCAAAGATGCAAGCGTTAATACGTATACAGCGaattcgcg-3’. The underlined nucleotide sequence denotes an idealized PPARγ response element motif. Streptavidin-coupled Dynabeads were purchased from Invitrogen. The oligos were synthesized with biotin attached at both the 3’- and 5’-ends. About 20–30 million cells were used per pull-down per condition.

Immunoprecipitation—Cultured ΔL1 cells (15-cm dish) were washed twice with 1×PBS buffer before adding 1 ml of prechilled Lysis Buffer (Pierce: PI87788) containing phosphatases and proteases inhibitors. Cells were removed from the plate with a disposable spatula, transferred into a 15-mi Falcon tube, and incubated on ice for ~20min. The lysate was then centrifuged at 4k rpm for 5min to remove the nuclear pellet. Because of excess of lipids, the lysate was filtered through a 0.45 μm spin-column (Millipore: Cat. 20–218) and pre-cleared with 15 μl of Protein A/G magnetic beads before adding 3 μg of mouse monoclonal anti-human PPARγ or mouse IgG to the samples for overnight incubation on a rotator at 4°C. The beads were washed 2–3 times with 1 ml of cold 1× PBS the following day. The IP samples were validated by WB before submitting it to mass spectrometry analysis. About 10–20 million cells were used per pull-down per condition.

Mass Spectrometry—Samples were boiled at 95°C for 10–15 min, before loading onto 4–20% gradient SDS-PAGE at 100V for 30 min (supplemental Fig. S2B). The gel was Coomassie stained for 1h at room temperature with shaking. Three bands were cut per lane/ sample and separately prepared for in-gel digestion as follows. After bands were destained and dehydrated, they were treated with 10 mM DTT for reduction of cysteines and 50 mM iodoacetamide for alkylation before subjecting them to porcine trypsin (Promega V5111) digestion overnight at 37°C. Before mass spectrometry analysis, the peptide pools were acidified, desalted through Zip-Tip C18 tip columns and dried down. Each sample was reconstructed in 100 μl of 0.1% formic acid and 13 μl were loaded into the system. The samples were analyzed by liquid-chromatography-tandem MS (LC-MS/MS) using an EASY-nLC 1000 system coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific). Peptides were concentrated and desalted on an RP precolumn (0.075 × 20 mm Acclaim PepMap 100 nano Viper, Thermo Fisher Scientific) and on-line eluted on an analytical RP column (0.075 × 150 mm Acclaim PepMap RSLC nano Viper, Thermo Fisher Scientific), operating at 300 nI/min using the following gradient: 5% B for 3 min, 5–40% B in 60 min, 40–80% B in 10 s, 80% B for 5 min, 80–5% B in 10 s, and 5% B for 11:40 min [solvent A: 0.1% formic acid (v/v); solvent B: 0.1% formic acid (v/v), 80% CH3CN (v/v)] (Fisher Scientific). The Q Exactive was operated in a data-dependent MS/MS mode using the 10 most intense precursors detected in a survey scan from 380 to 1600 m/z and performed at 70K resolution. Tandem MS was performed by HCD fragmentation with normalized collision energy (NCE) of 27.0%.

Raw data were converted to *.mgf files using Thermo Proteome Discover v2.1. Protein identification was carried out using both Sequest and Mascot programs against the in-house database generated from the UniProt/SwissProt mouse proteome. Oxidation (Met) and phosphorylation (Ser, Thr) were selected as variable modifications. Other settings included were carbamidomethylation of Cys as fixed modification, three missed cleavages, and a mass tolerance of 10 and 20 ppm for precursor and fragment ions, respectively. Saffold software (Proteome Software, Inc., Portland, OR) was used for evaluation and analysis where the protein and peptide thresholds were set at 95% with a minimum of two unique peptides per protein.

Experimental Design and Statistical Rationale—Both IP and affinity-tagged oligo pull-downs were performed with two biological replicates for all PPARγ IPs of the cytosolic fraction with 4-day ligand treatment and the nuclear fractions for DMSO, RSG, and SR255 treatments. We focused on ligand treatments with RSG and SR255 because although SR1664 is a well-characterized antagonist for PPARγ, its use as a chemical probe in animal models is limited because of its poor pharmacokinetic properties. Samples with more than one biological replicate analysis are indicated in supplemental Table S1. For comparative analysis of identified proteins, nondifferentiated MEFs were utilized as a negative control for the IP analysis. In the case of oligo trap experiments, three controls were performed, nondifferentiated MEFs, precleared PPARγ, and scrambled oligo, to aid data analysis.

Protein Expression—Protein expression and purification were performed similarly to that previously described (33, 34). Full-length WT Flag-hRXRα and WT Flag- hRXRβ were expressed in a baculovirus system and purified by Flag-SEC, respectively. Full-length WT HisTag-hPPARγ was expressed in E. coli BL21(DE3) and purified by HisTrapp FF column (GE Healthcare). The final protein buffer was 50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol and 2 mM DTT. The purity of each protein was >95% and was verified using SDS-PAGE, WB, and MALDI MS.

Hydrogen-Deuterium Exchange (HDX) Detected by Mass Spectrometry (MS)—Solution-phase amide differential HDX experiments were carried out with a fully automated system (CTC HTS PAL, LEAP Technologies, Carrboro, NC; housed inside a 4°C cabinet) as described previously with slight modifications (35). Peptides were identified using tandem MS/MS experiments performed with QExactive (Thermo Fisher Scientific, San Jose, CA) over a 70-min linear gradient. Product ion spectra were acquired in a data-dependent mode and the five most abundant ions were selected for the product ion analysis per scan event. The MS/MS*.raw data files were converted to *.mgf files and then submitted to MASCOT (version 2.3 Matrix Science, London, UK) for peptide identification. The maximum number of missed cleavages was set at 4 with the mass tolerance for precursor ions ≤ 0.6 Da and for fragment ions ≤ 8 ppm. Oxidation to Methionine was selected for variable modification. Pepsin was used for digestion and no specific enzyme was selected in the MASCOT during the search. Peptides included in the peptide set used for HDX detection had a MASCOT score of 20 or greater. The MS/MS/MASCOT search was also performed against a decoy (reverse) sequence and false positives were ruled out if they did not pass a 1% false discovery rate. The MS/MS spectra of all the peptide ions from the MASCOT search were further manually inspected and only the unique charged ions with the highest MASCOT score were included in the HDX peptide set.

HDX of full-length PPARγ, RXRα, RXRβ, and the duplex nonbiotinylated half-site DR1 (5’-AAAAACTAGG7CA-3’) DNA oligo in 50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol and 2 mM DTT was performed at 4°C using an automated system described previously (36). Proteins and DNA samples were run at 10–12 μl. Specifically, PPARγ, RXR, DNA, and ligand were mixed at 1:1:1:2:10 molar ratio, respectively. Briefly, proteins and DNA were incubated at 4°C in a D2O buffer for a range of exchange times (0s, 10s, 30s, 60s, 900s, and 3600s) before quenching the deuterium exchange reaction with an acidic quench solution (pH 2.4) containing 3 M Urea and 1% TFA.
Protein digestion was performed in-line using an immobilized pepsin column. Mass spectra were acquired on a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific).

HDX analyses were performed in triplicate, with single preparations of each protein/complex. The intensity weighted mean m/z centroid value of each peptide envelope was calculated and subsequently converted into a percentage of deuteron incorporation. This is accomplished by determining the observed averages of the undeuterated and fully deuterated spectra and using the conventional formula described elsewhere (37). Statistical significance for the differential HDX data was determined by an unpaired t test for each time point, a process integrated in the HDX Workbench software (38).

RESULTS

The compounds used in the studies presented here are well characterized and represented distinct pharmacology from each other; a full agonist rosiglitazone (RSG, $K_d$ ~20–80 nM), a partial agonist MRL24 ($K_d$ ~10 nM), an antagonist SR1664 ($K_d$ ~100 nM), and an inverse agonist SR2595 ($K_d$ ~100 nM) (1, 32). Although the pharmacological effects of these compounds have been previously described, their influence on the isoform of PPARγ has not been reported.

Detection of PPARγ1 and PPARγ2—The coexistence of the PPARγ1 isoform (28 residues shorter at the N terminus) and PPARγ2 has been described several times in previous studies involving ΔL1 cells (39–43), yet it is unclear what the composition of the two distinct bands shown in many published Western blots (44–46). To address this, full-length recombinant human PPARγ2 was expressed and purified to be used as a Western blot (WB) control for evaluation of the selective PPARγ2 antibody used in these studies (supplemental Fig. S1). WB analysis of nuclear and cytosolic fractions from both IP and 3xDR1 pull down, suggests that PPARγ1 was not detected. The epitope corresponding to PPARγ2-only antibody and the generic PPARγ antibody used for IP is shown in supplemental Fig. S1. Previous publications suggest the band directly below the PPARγ2 band (aligned near the 50kDa referenced marker) is typically referred to as PPARγ1 because it is ~3 kDa lower in mass. However, another possibility is that this band is related to antibody cross-reactivity or detection of the heavy chain of IgG because this same band was observed in the IgG, 3xDR1 pull-down (no antibody), and preadipocyte IPs (MEF) (Fig. 1 and supplemental Fig. S1). WB analysis of IgG and preadipocytes should not result in detection of either isoform of PPARγ, a notion that is supported by the MS/MS data of these samples. In addition, multiple gel bands from pull-down experiments were excised in the region of 40–50 kDa digested with trypsin, and subjected to MS/MS analysis (See supplemental Fig. S2C). The three most abundant proteins detected were actin (42 kDa), tubulin beta-5 chain (50 kDa), and elongation factor 1-alpha (50 kDa); proteins that have 42–46% sequence similarity to PPARγ based on LALIGN algorithm analysis. Further support that the lower band is not PPARγ includes a study where authors abrogated ΔL1 cells with a specific siRNA, and the lower band was still observed (43). Also, as shown in supplemental Fig. S1C, this lower band was detected in mock-transfected 293T cell lysates from a commercial vendor, Santa Cruz Biotechnology, Inc. Conversely, PPARγ2 was found unambiguously in both cellular fractions using either PPARγ IP or 3xDR1 trapped (supplemental Fig. S1A and S1B, red band). Based on these observations, we can conclude that only PPARγ2 was expressed in ΔL1 cells at levels sufficient to allow isolation and detection.

Ligand-dependent Effects on PPARγ Expression and Cellular Localization—Differentiation of MEF 3T3-L1 cells (ΔL1) was induced for 2 days and then cells were exposed to ligands for 4 days (Scheme 1). Cells were lysed and fractionated per Pierce Technology Inc. protocol. To assure the integrity of subcellular isolation, WB analysis of known regional proteins was performed and these results are shown in supplemental Fig. S3, where Gapdh was not observed in the nuclear fractions and histone 3, was only observed in the insoluble nuclear fraction. Although there were low levels of Hsp90 in the soluble nuclear fraction (NEB), this was expected and is consistent with the vendor’s results (supplemental Fig. S3B). WB analysis of immunoprecipitated (IP’ed) PPARγ from both the cytosolic and nuclear fractions from these cells showed that ligand exposure impacted the level of PPARγ protein (Fig. 1A). RSG, a full agonist, robustly increased the levels of PPARγ protein more than that observed with partial agonist MRL24 treatment whereas exposure of cells to the inverse agonist SR2595 reduced the levels of PPARγ protein below that observed for cells treated only with DMSO. This gradient of ligand-induced protein expression is markedly apparent in the nuclear fraction whereas less obvious in the cytosolic fraction, perhaps reflecting the change in the equilibrium between PPARγ translation and translocation (Fig. 1A). These observations are consistent with previously published qPCR data in which PPARγ mRNA expression level following RSG, MRL24, and SR2595 treatment was 4-fold, 2-fold, and 0.85-fold, respectively, relative to DMSO (1, 32).

DNA Oligo Trap of PPARγ in the Nucleus and Cytoplasm of Adipocytes—The oligo pull-down experiments were performed on both the cytosolic and soluble nuclear fraction following 1-day compound treatment. In this experimental paradigm PPARγ protein level is at steady-state (Fig. 1B). As such 1-day ligand treatment of ΔL1 cells would reflect the effects the compounds have on the PPARγ interactome within adipocytes independent of their effects on expression of the receptor. As shown in Fig. 1A, prolonged treatment with antagonists or inverse agonists (e.g. SR1664 or SR2595) leads to reduced levels of PPARγ.

Three controls were incorporated into the experimental design; (1) a biotinylated scrambled oligo (ScrO), (2) lysate where PPARγ was immuno-depleted (XPPARγ), and (3) lysate from undifferentiated MEFs. These controls were used to identify proteins that bind to the oligo in the absence of PPARγ and are considered nonspecific interactions (supplemental Fig.
Validation of the controls was performed as follows. PPARγ/H9253 was not detected by WB and MS/MS analysis following pull-down from lysates with the ScrO. However, WB analysis of the XPPARγ/H9253 lysate revealed the presence of very low levels of PPARγ/H9253 (supplemental Fig. S4A). MSMS analysis of the XPPARγ/H9253 lysates from cells treated with DMSO and RSG resulted in detection of only one unique PPARγ/H9253 peptide and no PPARγ specific peptides were detected in the XPPARγ/H9253 samples from cells treated with MRL24, SR1664, and SR2595. This is contrasted to the 11–18 unique peptides detected in lysates that were not immuno-depleted (supplemental Table S1C), suggesting efficient preclearing of PPARγ before oligo pull-down. PPARγ-specific peptides were not detected in MEF lysates.

**Nuclear PPARγ Complexes Identified on 3xDR1**—The total number of protein IDs from pooled proteomic experiments are listed in supplemental Table S1A. An average of 290 unique proteins was identified in all experiments in which lysates were exposed to ScrO in contrast to ~205 unique proteins in lysates exposed to 3xDR1. The number of unique proteins reduced to an average of 135 proteins when the lysates were precleared of PPARγ (XPPARγ) before addition of the 3xDR1 oligo whereas exposure of undifferentiated MEF lysates to 3xDR1 resulted in the identification of an average of 170 proteins. Filtering out proteins identified in the three control experiments (ScrO, XPPARγ, and MEFs) from those observed in all of the 3xDR1 data sets including DMSO only and all ligand treatments (avg. 204 proteins), resulted in two common proteins, PPARγ and RXRα. These two proteins were unaltered by treatment with DMSO or ligands (Fig. 2 and supplemental Fig. S5) and required both 3xDR1 and PPARγ to be pulled down. As such, these proteins are considered part of the nuclear PPARγ complex independent of ligand. The presence of PPARγ confirms the selectivity of the enrichment strategy and the presence of RXR was anticipated. Interestingly, whereas all three RXR isoforms are expressed in H9004L1 cells (data not shown), only RXRβ was found to be PPARγ:DR1 specific (the RXR isoform specific peptides detected in

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**Fig. 1. Biotinylated DNA oligo capture and PPARγ IP in different cellular environments of H9004L1 cells.** A) PPARγ IP in the cytoplasm and nucleus following 4-day ligand treatment using mouse monoclonal anti-human PPARγ antibody (m_hPPARγ). IgG control is shown in the middle. B, and C, 3× DR1 biotinylated oligo capture in the nucleus and cytoplasm following 1-day ligand treatment, respectively. D) PPARγ IP in the nucleus following 1-day treatment. The SDS-PAGE gels were transferred to nitrocellulose and proteins were visualized using rabbit polyclonal anti-human PPARγ antibody (r_hPPARγ), and a fluorescent labeled secondary antibody from rabbit to detect the respective IgG isotype. Band A indicates endogeneous PPARγ from H9004L1 cells where band B denotes human Flag-tagged PPARγ2 transfected in the HEK293T cell line. The arrow that is marked with a (?) is likely because of cross-reactivity with the antibody rather than PPARγ1 isoform. WBs were processed and analyzed using Licor Technology.
Ligand Modulation of the PPARγ Interactome

FIG. 2. Venn diagrams of the nuclear interactome complexes of the 3xDR1 capture following 1-day ligand treatment. Proteins listed were identified by MS/MS pertaining to specific ligand treatment and post filtering with the respective controls (MEFs, XPPARγ, and ScrO). The two common proteins are PPARγ and RXRβ where the double asterisk indicates proteins that were observed in both the nuclear and cytosolic fractions. DMSO, RSG, and SR2595 data were extracted from two biological replicates.

the MSMS data sets are listed in (supplemental Table S2). For instance, RXRα was detected in lysates of both MEF and XPPARγ exposed to 3xDR1 but not when exposed to ScrO, suggesting its interaction with the 3xDR1 oligo is specific but independent of PPARγ. In contrast, RXRγ was observed only in the DMSO and RSG treated samples exposed to 3xDR1.

Ligand-dependent Associated Proteins on 3xDR1 in the Nucleus—Post filtering, the DMSO-treated cells, which should contain PPARγ bound to its endogenous ligand, had four unique proteins identified (~41). MRL24 treated samples had the most number of uniquely identified proteins at thirteen. In addition to PPARγ, other NRs were identified. ERR1 (or ESSRA) was observed in cells treated with the antagonist SR1664 and RARγ was observed in cells treated with the inverse agonist SR2595, suggesting these NRs might be components of repressive PPARγ complexes. Other proteins, such as RD23B, were observed in all ligand treated samples except cells treated with SR1664. HLTF, TF7L2, and TIM50 were only identified in the RSG and MRL24 treated samples. Interestingly, TF7L2 had been shown to be associated with T2D (47). Some of the proteins that were excluded from the Venn diagram displayed in Fig. 2 include the nuclear receptors NR2C1 (TR2), NR2C2 (TR4), and NR2F6 (EAR-2), because they were detected in the nuclear fraction of undifferentiated MEFs suggesting they bind to 3xDR1 independently of PPARγ. NR coregulatory proteins such as DDX5, FLNA, NONO, RAN, XRCC5, and XRCC6 (www.nursa.org) were also excluded because they were detected in one or more control samples. C/EBPα and C/EBPβ, which are transcriptional regulators of PPARγ expression, were also filtered out as they were only observed in the ScrO samples.

Cytosolic PPARγ Complexes Identified on 3xDR1—The same strategy described above was applied to extract PPARγ complexes from the cytosolic fraction of lysates. The rationale for this was to determine if there are conserved components associated with PPARγ in both fractions. Unique cytosol complexes could be indicative of protein interactions that influence receptor translocation, or perhaps an alternative cytosolic function for PPARγ like that observed for VDR (48). As shown in Fig. 1C and (supplemental Table S1A), the ScrO controls had the most proteins (avg. 786 proteins), followed by 3xDR1 (avg. 640 proteins), XPPARγ (avg. 242 proteins), and MEFs lysates with an average of 331 proteins. After filtering out proteins detected in the controls from each ligand treatment data set, five unique NRs were found to be coisolated with PPARγ independent of the ligand used (supplemental Fig. S6). Similar to that observed in the nuclear fraction studies, RXRβ was the only RXR isoform detected to be PPARγ-dependent (Fig. 3). RXRα was observed in all XPPARγ control samples but not in the ScrO samples nor in the MEF lysates further supporting that RXRα binds specifically to 3xDR1, but independent of PPARγ. In contrast, detection of RXRβ on 3xDR1 requires the presence of PPARγ suggesting an alternative model in which RXRβ is an obligate coreceptor for PPARγ in fat cells and selectively interacts with PPARγ:3xDR1. Interestingly, RXRγ was only detected in the nuclear fraction when cells were treated with RSG or DMSO only (cells where PPARγ should be bound to its endogenous ligand), suggesting it is both PPARγ-specific and ligand-dependent.

In addition to RXRβ, nine other PPARγ-dependent proteins were detected in both subcellular compartments (see supplemental Table S1D). These two proteins, HLTF and RD23B, are of interest. HLTF is a helicase belonging to the SWI/SNF family and thought to be involved in regulation of gene transcription. RAD18, an isoform of RD23B and a UV excision repair protein that plays a role in DNA repair, has been shown to interact directly with HLTF (49). Interestingly, these two proteins were not observed when cells were treated with the antagonist SR1664.

Probing Conformational Changes of PPARγ Affected by RXRα and RXRβ Via HDX-MS—Although the controls were designed to determine proteins dependence on PPARγ, the 3xDR1 oligo sequence is homologous to the cytoplasmic retinol binding protein II RXRα response element (CRBP) that can recruit both RARα and RXRβ (50). Considering RXRβ is a critical element in transcriptional regulation and has been previously shown to interact with PPARγ in yeast two-hybrid screens (51), we applied hydrogen/deuterium exchange mass spectrometry (HDX-MS) to probe the interaction of PPARγ with RXRβ and compared it to the obligatory heterodimer of RXRα/PPARγ in the presence of DNA oligo. As shown in Fig. 4, in the presence of either RXRα or RXRβ, the conformational dynamics of the PPARγ DBD were stabilized on binding to a
DR1 indicated by protection to solvent exchange whereas in the absence of DNA, there were minimal changes when PPARγ was bound to either RSG or SR2595 ligands (Fig. 4, panels 4A–4D, red curves). PPARγ binding to RXRα induced a greater magnitude of protection to solvent exchange within the DBD zinc finger region as compared with RXRβ (blue dashed line of Fig. 4). Further, binding to the antagonist SR2595 induced more protection to the helical region of the receptors DBD, a region critical to binding Zn(II) (residues LAVGMSHARF) than did the RSG (Fig. 4, blue dashed lines of panel C versus panel 4A). These data suggest the RXR isoforms might differentially influence how PPARγ binds to specific response elements which in turn would impact the level of transcriptional output for a subset of PPARγ target genes.

Additionally, binding of SR2595 to PPARγ/RXRα heterodimer shielded the DBD region from solvent exchange to a greater extent when compared with the complex bound to the full agonist RSG, suggesting that ligand binding can also influence receptor interaction with DNA.

**PPARγ Interactome by IP is Distinct from Oligo Trap in Adipocytes**

Nuclear PPARγ Complexes Identified by IP—Immunoprecipitation of PPARγ in subcellular fractions of cells treated with various ligands was performed and the observed interactomes were compared with the 3xDR1 results. Western blot analysis and the number of proteins and PPARγ peptides identified following IP with a PPARγ-specific antibody or IgG of lysates from ligand-treated 3T3-L1 cells and undifferentiated MEFs are shown in Fig. 1D; supplemental Table S1B and S1C. Compared with the data sets from oligo pull-downs, fewer proteins were identified following IP and the total number of proteins varied across the different ligand treatment groups. For instance, IP of RSG treated cells resulted in detection of about 100 proteins whereas over 200 proteins were detected using 3xDR1. The most striking observation was that different interactomes were detected using IP when compared with 3xDR1 pulldowns. After filtering out proteins that were detected following treatment with control IgG and those that were detected in the MEFs (supplemental Fig. S7), the consensus unique proteins associated with each ligand treatment have no overlap with those detected using 3xDR1. One possible explanation for this is that the antibody is only capable of precipitating non-DNA bound PPARγ and this population of the receptor has distinct properties from PPARγ bound to DNA. Supporting this notion are studies that have shown DNA alters the conformational dynamics of NRs and DNA binding likely alters the proteins that PPARγ interacts with (Fig. 5) (33, 52). Overall, only two proteins were found to be common in IP data sets from all ligand treatments - PPARγ and RL31. In contrast to that observed in the 3xDR1 experiments, no NR was detected in the IPs, suggesting that the interaction of PPARγ with other NRs is DNA-dependent.

Some of the known regulators found in RSG, MRL2, and SR1664 are DDX5, PHB, and SFPQ and BAZ1B, respectively. However, none of these were observed in the oligo pull-downs, suggesting they might not be involved in PPARγ transcriptional regulation. A closer look at proteins unique to MRL24 treatment using Ingenuity Pathway Analysis (IPA) showed that majority of them are associated with EIF2 signaling pathway.

**Cytosolic PPARγ Complexes Identified by IP**—To explore the PPARγ interactome in the cytosol, PPARγ was IP’ed from the cytosolic fraction following treatment of cells with ligands for 24 h. In stark contrast to that observed in the nuclear fraction, the cytoplasmic PPARγ interactome was not altered when comparing DMSO treatment to other compounds. Filtering out proteins detected in IP of MEFs and IgG control resulted in detection of approximately the same number of PPARγ peptides being detected, ranging from 10 to 13 (sup-
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Fig. 4. HDX analysis of PPARγ conformational changes in the presence of RXR isoforms, DNA, and ligands. The blue and red curves (panels A–D) correspond to receptor with and without DNA, respectively. Helical regions of PPARγ DBD consisting of LAVGMSHNAIRF (blue color) and RVCGDKASGFHYGVHACEGCKGF (green color) are mapped onto PDB:3DZY for PPARγ/RXRα/DNA complex. All the experiments were performed in triplicate.

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Ligand-dependent Protein Association in the Cytoplasm Detected by IP—Comparison of the filtered data sets from all ligands for 4 days resulted in a varied number of PPARγ peptides detected (ranging from 5 to 14 peptides (supplemental Table S1C). This is not surprising considering SR2595 is a partial inverse agonist that represses PPARγ expression. After filtering proteins detected in controls, the DMSO and RSG treated samples resulted in 6–8 unique proteins whereas cells treated with MRL24 and SR2595 resulted in 48–56 unique proteins (supplemental Fig. S9). The large variation in the number of unique proteins detected does not correlate with the total amount of PPARγ in each sample. As expected and shown in Fig. 1A, WB analysis showed that both the RSG and MRL24 treated cells (agonist and partial agonist) have more PPARγ protein than the DMSO and SR2595 treated cells (endogenous ligand and partial inverse agonist). These data suggest that the PPARγ interactome is modulated directly by its ligand.

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4-day compound treatments (Fig. 6 and the attached Excel file in SI) resulted in 3 unique proteins for DMSO, 5 for RSG, 33 for MRL24 and 40 for SR2595 treatment. Many of these proteins are enzymes involved in the metabolic pathways. For instance, dihydrolipoyl dehydrogenase (DLDH) is known to be regulated by RSG and both APMAP and PLPL2 are associated with lipogenesis. Based on IPA, several proteins in the MRL24 were grouped in the acetyl-CoA biosynthesis pathway and those in SR2595 were associated with carbohydrate metabolism. In general, the proteins observed are relevant to adipocyte function. Perhaps the most striking observation are the proteins unique to DMSO, MRL24 and SR2595 treatment but absent in the RSG treated samples (supplemental Table S3). Analysis of this subset of proteins shows that a majority of them are associated with the extracellular matrix. Functional and pathway analysis using IPA reveals that these proteins are associated with cellular assembly, function and organization, which if dysregulated, can lead to skeletal and muscular disorders, including cardiovascular disease. This is of significant interest because TZD use in humans is associated with an increase in cardiovascular events.

**DISCUSSION**

The overall goal of this study was to qualitatively compare alterations of the PPARγ interactome in two distinct cellular compartments (cytosol and nucleus) on treatment of A1 cells with a set of pharmacologically distinct ligands. Additionally, we sought to compare the interactomes detected using traditional antibody-based IP from that observed using oligo trapping. Results presented here demonstrate the ability of a short DNA oligo representative of an idealized tandem PPARγ response element (3xDR1) to extract endogenous PPARγ from the two subcellular compartments, and the major PPARγ isoform expressed in A1 cells is PPARγ2. However, significant differences were observed when comparing the PPARγ interactome obtained by IP to that from 3xDR1 pull-down suggesting that the PPARγ complex is different when the receptor is bound to DNA.
Which RXR Isoform is the Coreceptor for PPARγ?—Because most RXR ChIP-Seq analysis studies were performed using pan RXR antibodies to determine cooccupancy with PPARγ, it is impossible to learn from these data sets if RXRβ has been found to be associated with PPARγ. Experiments performed with 3xDR1 pull-down revealed unexpectedly that RXRβ may be the preferred RXR isoform in the context of the PPARγ/RXR heterodimer bound to chromatin (Fig. 7). Because both RXRα and RXRβ have similar expression levels in L1 cells, perhaps there might be competition between RXRα and RXRβ to interact with PPARγ, and the 3xDR1 used here favors RXRβ interaction. Whereas the canonical PPRE oligo implemented here represents the consensus DR1 and is not specific for any one target gene, we have shown here that RXRβ is highly specific to PPARγ. Furthermore, HDX studies reveal unique conformational dynamics in the DBD of PPARγ when comparing PPARγ/RXRα to PPARγ/RXRβ on interacting with DR1. These unique conformational dynamics persists in the presence of either agonist (RSG) or inverse agonist (SR2595). Additional studies are required to determine if RXRα and RXRβ control unique nonoverlapping sets of PPARγ target genes.

PPARγ/NR Interactions is Highly DNA Dependent—An increasing number of studies have shown that NRs can act as coregulators of other NRs. For example, PR has been shown to drive ERα activity in the context of breast cancer (53), sumoylated PPARγ functions as a coregulator to transrepress NF-κB target genes by tethering in NCOR1 (54), and RXRβ has been shown to coregulate RARα, TR, and VDR (50). Thus, the NRs observed in the pull-downs described here could be direct modulators of PPARγ activity by serving as coreceptors within the heterodimer (e.g., RXRα and RXRβ) or they could interact with PPARγ AF2 surface, or by interaction with the N-terminal domains of the PPARγ:RXR:DNA complex (e.g., RARγ and ERR1).

The interaction of ERR1 with PPARγ bound to SR1664 is interesting as ERR1 is an orphan receptor and has been implicated in bone loss and osteoporosis. A 2013 study demonstrated that in vivo ERR1 undermined the commitment of mesenchymal stem cells (MSCs) to differentiate into osteoblasts during resulting in reduced bone mineral density (55). A subsequent study demonstrated that conditional knockdown of ERR1 protected mice from bone loss. In our studies, mRNA transcriptional level of ERR1 was 5-fold higher in SR1664

![A working model of PPARγ interactome networks with suggested key components.](image-url)
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treated cells in relation to the DMSO treated cells (data not shown). Likewise, RARγ was only observed to be associated with PPARγ in the nucleus of cells treated with the inverse agonist SR2595, suggests it might associate with PPARγ repressive complexes. BioGRID database cites RARγ as having interactions with RXRβ, NR2F6, and RXRα in yeast two-hybrid (2YH) assays (56) suggesting RARγ could be a coregulator of RXRβ:PPARγ heterodimers. All three RAR isoforms (α, β, γ) do express in ΔL1 cells (data not shown), but only RARγ was observed in replicated experiments.

Non-DNA Bound PPARγ Interactome is Different from DNA Bound PPARγ Interactome—The PPARγ interactome in both subcellular compartments was found to be completely different between that obtained using antibody IP from that using 3xDR1 pull-down. Among the hundreds of proteins detected, only three proteins overlapped between the two methods, suggesting that PPARγ interacts with a different set of proteins depending on whether it is bound to DNA or not. Although other NRs were found to be associated with PPARγ using oligo trapping, none were detected in either subcellular compartment when the receptor was IP’ed with an antibody, including the RXRs. Likewise, IP of RSG treated cells using an RXR antibody did not result in detection of PPARγ (data not shown), further supporting that the interactions of RXRs and other NRs with PPARγ is DNA-dependent.

The Effect of Agonism on Bone Density and Lipid Accumulation—Analysis of mRNA expression levels of fpps by qPCR demonstrated that RSG and MRL24 increase its expression by 18-fold and 3-fold, respectively, when compared with DMSO only. The expression level of fpps was unchanged following treatment of cells with SR2595. These data suggest that the negative effects on bone density following TZD use could be associated with PPARγ modulation of fpps levels (supplemental Fig. S2). Interestingly, SR2595 had recently been shown to have a beneficial effect on osteogenesis (1). Other proteins of interest that were unique to RSG treatment include APMAP (+7-fold in mRNA level) which plays a role in adipocyte plasma membrane homeostasis and PLPL2 (+48-fold in mRNA level), which is involved in fatty acids catalysis. It is likely that these proteins are associated with TZD effects on adipocyte hypertrophy and increased adiposity observed in patients. Perhaps the most striking observation was made in the IP samples of the cytoplasmic fraction where proteins unique to DMSO, MRL24 and SR2595 treatment are absent in the RSG treated samples (supplemental Table S3). Analysis of this subset of proteins shows that most of them are associated with the extracellular matrix. Functional and pathway analysis using IPA reveals that these proteins are associated with cellular assembly, function and organization, which if dysregulated, can lead to skeletal and muscular disorders, including cardiovascular disease. This is of significant interest because TZD use in humans is associated with an increase in cardiovascular events.

CONCLUSION

Using proteomics and affinity enrichment through two different mechanisms to capture both DNA-bound and non-DNA bound PPARγ, these studies have provided insight into the dynamics of the PPARγ interactome in the context of cellular localization and ligand status. Results are suggestive of differences observed in animal models with these pharmacological distinct ligands. Of interest is the detection of specific binding of RXRβ to PPARγ in the presence of DNA. Subsequent HDX studies suggest that RXR isoforms differentially alter the conformational dynamics of PPARγ in a key region of its DBD. These unique changes could potentially impact PPARγ target gene expression.

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DATA AVAILABILITY

The mass spectrometry proteomics data had been deposited to PeptideAtlas (www.peptideatlas.org) with the designated dataset identifier PASS01006.

This article contains supplemental material.

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