Nuclear Receptor-Coregulator Interaction Profiling Identifies TRIP3 as a Novel Peroxisome Proliferator-activated Receptor γ Cofactor*§

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Nuclear receptors (NRs) are major targets for drug discovery and have key roles in development and homeostasis as well as in many diseases such as obesity, diabetes, and cancer. NRs are ligand-dependent transcription factors that need to work in concert with so-called transcriptional coregulators, including corepressors and coactivators, to regulate transcription. Upon ligand binding, NRs undergo a conformational change, which alters their binding preference for coregulators. Short α-helical sequences in the coregulator proteins, LXXLL (in coactivators) or LXXXIIIXXL (in corepressors), are essential for the NR-coregulator interactions. However, little is known on how specificity is dictated. To obtain a comprehensive overview of NR-coregulator interactions, we used a microarray approach based on interactions between NRs and peptides derived from known coregulators. Using the peroxisome proliferator-activated receptor γ (PPARγ) as a model NR, we were able to generate ligand-specific interaction profiles (agonist rosiglitazone versus antagonist GW9662 versus selective PPARγ modulator telmisartan) and characterize NR mutants and isoforms (PPARα, -β/δ, and -γ). Importantly, based on the NR-coregulator interaction profile, we were able to identify TRIP3 as a novel regulator of PPARγ-mediated adipocyte differentiation. These findings indicate that NR-coregulator interaction profiling may be a useful tool for drug development and biological discovery. Molecular & Cellular Proteomics 8:2212–2226, 2009.

Nuclear receptors (NRs)1 are ligand-inducible transcription factors involved in development and homeostasis that play key roles in many diseases, including diabetes, cancer, and obesity (1). NRs consist of several functional domains, which exhibit varying degrees of conservation among members of the receptor family. The poorly conserved N terminus contains the activation function 1 (AF-1) domain, the activity of which is often regulated by post-translational modifications. Centrally located is the DNA binding domain, which is highly conserved among species and between nuclear receptors. The ligand binding domain (LBD), which is also relatively well conserved in terms of primary amino acid sequence, mediates ligand binding, and contains the powerful ligand-dependent activation function (AF-2). LBD crystal structures have revealed a canonical fold consisting of 13 α-helices and a small four-stranded β-sheet (2). Upon ligand binding, the AF-2 helix (also referred to as helix 12) is stabilized in an active state (3). Depending on the conformation of the LBD and its modulation by ligand, NRs can recruit or release transcriptional coregulator proteins that perform all of the subsequent reactions needed to induce or repress transcription of target genes (4). Coregulators are often components of large multiprotein complexes that act in a sequential and/or combinatorial fashion to modify chromatin and to recruit basal transcription factors and RNA polymerase II (5). In general, the transcriptional coregulator family consists of coactivators, which associate with active, liganded receptors and corepressors, which interact with inactive, unliganded (or antagonist-bound) receptors. Short peptide motifs within coactivator and corepressor proteins are responsible for their overlapping but non-identical binding to the LBD surface. LXXLL motifs (where L is leucine and X is any amino acid) are found in many coactivator proteins (6), whereas LXXXIIIXXL motifs (where I is isoleucine) are present in most corepressor molecules (7). Both motifs probably form an amphipathic α-helix upon binding to the hydrophobic cleft on the surface of the LBD (8).

The NR superfamily includes the closely related peroxisome proliferator-activated receptor family. PPARs) α, β/δ, and γ. Although all three PPARs participate in lipid and glucose metabolism, their roles in these processes are complex and context-dependent. PPARγ, in particular, has been extensively studied due to its role in lipid metabolism and insulin resistance. PPARγ is activated by thiazolidinediones (TZDs), such as rosiglitazone (Avandia®) and pioglitazone (Actos®), which are used to treat type 2 diabetes. PPARγ activation leads to the expression of genes involved in lipid metabolism, glucose uptake, and inflammation. The PPARγ-ligand complex interacts with coactivators, such as SRC-related kinases (SREKs) and nuclear receptor corepressor (NCoR), to mediate these effects. The study of PPARγ and its coregulators is crucial for understanding the molecular mechanisms underlying metabolic diseases and developing therapeutic strategies.

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1 The abbreviations used are: NR, nuclear receptor; AF, activation function; EC50, half-maximal effective concentration; FPLD3, familial partial lipodystrophy type 3; LBD, ligand binding domain; MI, modulation index; PPAR, peroxisome proliferator-activated receptor; siRNA, small interfering RNA; SNRM, selective NR modulator; SPPARγ-M, selective PPARγ modulator; TRIP, thyroid hormone receptor-interacting protein; TZD, thiazolidinedione; CBP, cAMP-response element-binding protein (CREB)-binding protein; PRIP, peroxisome proliferator-activated receptor-interacting protein; IKBB, IκBβ; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; NCoR, nuclear receptor corepressor; SHP, small heterodimer partner; SRC, steroid receptor coactivator.

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tabolism, the three isotypes exhibit different physiological roles due to (i) distinct expression patterns, (ii) specific activation by different ligands, and (iii) intrinsic functional differences between the different receptor proteins (9–11). PPARγ is the key regulator of adipocyte differentiation, maintenance, and function (12, 13) as exemplified by human familial partial lipodystrophy type 3 (FPLD3) patients. FPLD3 patients harbor heterozygous mutations in the PPARγ gene and are characterized by aberrant fat distribution and metabolic disturbances, including insulin resistance and dyslipidemia (11). Synthetic PPARγ agonists include the thiazolidinediones (TZDs), which ameliorate insulin resistance and lower blood glucose levels in patients with type 2 diabetes. Treatment of diabetic patients with synthetic PPARγ ligands of the TZD class, however, has been linked to adverse side effects like undesired weight gain, fluid retention, peripheral edema, and potential increased risk of cardiac failure (14, 15). These adverse side effects may be due to the use of high doses of full PPARγ agonists, suggesting that “activation in moderation” may be a more sensible approach (16). This may be achieved through the use of compounds displaying partial agonism, so-called selective PPARγ modulators (SPPAR-γMs) (17).

Given the broad range of diseases in which NR-based drugs are currently being used (18–20), high throughput profiling of interactions between a given NR and coregulator-derived peptides could be a very useful tool in drug development. Here we used peptide microarrays, as frequently used in enzymatic studies on kinases and proteases (21), to generate NR-coregulator interaction profiles. We generated ligand-specific interaction profiles (agonist, antagonist, or SPPAR-γM) and characterized NR mutants and isotypes (PPARα, -β/δ, and -γ). In addition, we identified a novel biologically relevant interaction between PPARγ and thyroid hormone receptor-interacting protein 3 (TRIP3). These findings indicate that NR-coregulator interaction profiling may be a useful tool for drug development and biological discovery.

EXPERIMENTAL PROCEDURES

**NR-Coregulator Interaction Profiling**—Assay mixtures were prepared on ice in a master 96-well plate with a 5 nm concentration of either commercial preparations of GST-PPARα-LBD, GST-PPARβ-δ-LBD, and GST-PPARγ-LBD (PV4692, PV4694, and PV4546; Invitrogen) or equivalent amounts of purified PPAR-γ LBD-GST wild type and mutants (see below), time-resolved fluorescence resonance energy transfer coregulator buffers J (for PPARα and -β/δ) and F (for PPARγ) (Invitrogen), 25 nM Alexa Fluor 488-conjugated anti-GST antibody (A11131, Invitrogen), 5 mM DTT, 2% DMSO, and ligand at the indicated concentration. All assays were performed in a PamStation®-96 controlled by EvolveHT software (PamGene International B. V., ’s-Hertogenbosch, The Netherlands) at 20 °C at a rate of 2 cycles/min. Nuclear Receptor PamChip® Arrays (PamGene International B.V.) contained 48 peptides (first generation array used in Fig. 1) or 53 peptides (second generation array used in Figs. 2–5). The arrays are made of a porous metal oxide surface that the

surface area is 500 times larger than that calculated based on spot diameter. A spot contains ~106 pores, each with a diameter of 0.2 µm and a length of 60 µm. In addition, pores are branched and interconnected. Arrays were incubated for 20 pump cycles with 25 µl of blocking buffer (1% BSA, 0.01% Tween 20 in Tris-buffered saline) and then aspirated. Per array, 25 µl of assay mixture was transferred from the master plate to the chip using a multichannel pipette. During the period of ligand incubation (~40 min), a solution of GST-PPARγ-LBD, fluorescent anti-GST antibody, and ligand was pumped through the porous peptide-containing membrane for 81 cycles at a rate of 2 cycles/min. Assay mixtures were incubated in the arrays for 80 cycles, and a .tiff format image of every array was obtained at cycles 21, 41, 61, and 81 by a charge-coupled device camera-based optical system integrated in the PamStation-96 instrument.

**Peptide Microarray Data Analysis**—Image analysis, consisting of automated spot finding and quantitation, followed by calculation of binding velocities was performed by Bionavigator software (PamGene International B.V.). In short, the boundaries of a spot were determined, and the median signal within the spot (signal) as well as that in a defined area surrounding it (background) were quantified. The signal-minus-background values were subsequently used for the calculation of the binding velocity. For each array and for each individual coregulator peptide, a binding curve of the NR to that coregulator motif was constructed from the binding at five consecutive time points using $y = y_0 + y_{\text{span}} \times (1 - e^{-t \times \text{offset}})$ (an exponential rise from level $y_0$ (at $x = \text{offset}$) to $y_0 + y_{\text{span}}$ (at $x \rightarrow \infty$) with rate constant $k$ ($k > 0$)). The derived slope (binding velocity) of the fitted curve (cutoff, $R^2 > 0.7$) at time point 31 was used as the parameter for interpretation. Ligand dose responses were analyzed with Prism for Windows 4.02 (GraphPad Software Inc., San Diego, CA) using non-linear regression, sigmoidal dose response (variable slope). Fitted dose-response curves delivered values for potency (half-maximal effective concentration (EC50)) and induction (difference in signal between the bottom and top value of the curve). EC50 values were presented only when a curve met the criterion of $R^2 > 0.5$. Induction values were presented only when the 95% confidence intervals of the bottom levels of the curve did not overlap with the 95% confidence interval of the top of the curve.

**NR-Coregulator Peptide Affinity Determination**—On a single array, a concentration range of the coregulator peptide was immobilized. $K_d$ values were determined from the binding of the NR at different peptide densities using non-linear regression with Prism for Windows 4.02 (sigmoidal dose response, variable slope, constrained, shared top).

**Plasmids**—The vectors for bacterial expression of GST-PPARγ-LBD, GST-PPARγ-LBDγ, GST-PPARγ-βδLBD, GST-PPARγ-αLBD, GST-PPARγ-βδLBD, and GST-PPARγ-αLBD were described previously as were the eukaryotic expression vectors pcDNA3-PPARγ2, pcDNA3-PPARγ2δ, and the 3xPRE-tk-Luc reporter (22–26). The pcDNA3-PPARγ2LBD constructs and verified by sequencing. pcDNA3.1-TRIP3 was purchased from GenScript Corp. (Piscataway, NJ), and pcDNA3.1-TRIP3 was generated by site-directed mutagenesis of the wild-type pcDNA3-PPARγ2 and GST-PPARγ-LBD γLBD construct and verified by sequencing. pcDNA3.1-TRIP3 was purchased from GenScript Corp. (Piscataway, NJ), and pcDNA3.1-TRIP3 was generated by site-directed mutagenesis using forward primers 5'-GATTCGAAAACTTTAAGAACGCGCCGGCTAAGACCCAC-3' and reverse primer 5'-GTGGGGTTAGCCGCCGCGTTCTTGAAGTTCCAGTTCGAC-3'.

**GST Protein Isolation**—Rosetta pLysS competent bacteria (Novagen, EMD Chemicals Inc., Darmstadt, Germany) were transformed with GST expression plasmids. GST fusion protein expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside and purified as described earlier (25). GST fusion proteins were eluted from glutathione-Sepharose beads (Amersham Biosciences) using elution buffer (20 mM glutathione, 100 mM Tris, pH 8.0, 120 mM NaCl).
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Proteins were concentrated using Vivaspun centrifugal concentrators (Sartorius, Epsom, UK), and protein concentrations were determined using SDS-PAGE followed by Coomassie Brilliant Blue staining.

**GST Pull-down Assays**—The full-length coding sequences of PPARβ/δ, PPARγ, TRIP3, and TRIP3m in the pcDNA3 or pcDNA3.1 expression vector were transcribed and translated in vitro in reticulocyte lysate in the presence of [35S]methionine (Amersham Biosciences) according to the manufacturer’s protocol (Tnt T7 Coupled Transcription/Translation kit, Promega, Madison, WI). 35S-Labeled proteins were incubated with GST fusion proteins in NETN buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing protease inhibitors (Complete, Roche Applied Biosciences). Samples were subsequently washed and subjected to SDS-PAGE. Gels were incubated with anti-Fabp4 (sc18661; Santa Cruz Biotechnology, Santa Cruz, CA), anti-PPARγL-068069-01 (23), and anti-tubulin (ab6046; Abcam, Cambridge, UK).

**Cell Culture and Reporter Assays**—The human osteosarcoma cell line U2OS was maintained in Dulbecco’s modified Eagle’s medium Glutamax containing 10% fetal calf serum (Invitrogen). Luciferase reporter assays were performed exactly as described previously (23) using 1 

### RESULTS

**PPARγ Agonists Induce Specific Coregulator Interaction Profiles**—The molecular determinants dictating NR-coregulator interactions are still largely unknown. To generate NR-coregulator interaction profiles, we used PamChip-96 peptide microarrays containing peptides of ~25 amino acid residues derived from known NR coregulator proteins (Table I) harboring either LXXLL (in coactivators) or LXXXIXXXL (in corepressors) motifs (see supplemental Table 1). The peptides are immobilized on a porous metal oxide carrier by piezo technology (Fig. 1a) as previously used in kinase assays (28–30). The peptide microarrays were incubated with NR LBDs by pumping the sample up and down through the three-dimensional metal oxide carrier in the absence or presence of ligand (Fig. 1, b and c). During each experiment images were collected at different incubation cycles using a PamStation-96 station, and a time-dependent increase of binding was clearly visible (Fig. 1c, lower panel). The spot signal intensity was plotted against cycle number (Fig. 1c, upper panel), and as a measure for binding the slope of the curve at cycle 31 ($V_{m}$) was used (Fig. 1c, upper graph). The use of $V_{m}$ values enhances the reliability of the interaction data, because they are calculated with more data points at cycles where binding has not reached saturation, resulting in a broader dynamic range (supplemental Fig. 1).

*Human Genome Organisation Gene Nomenclature Committee database symbol is in italic.*

### TABLE I

| Name         | Alternative name(s)* | Interaction with PPARγ | Ligand-dependent peptide interaction |
|--------------|----------------------|------------------------|--------------------------------------|
| CBP          | CREBBP, KAT3A        | 64                     | Yes                                  |
| p300         | EP300, KAT3B         | 64                     | Yes                                  |
| SRC1         | NCOA1, RIP160, KAT13A | 65                     | Yes                                  |
| TIF2         | NCOA2, GRIP1, SRC2, KAT13C | 66                     | Yes                                  |
| SRC3         | NCOA3, ACTR, AIB1, RAC3, TRAM-1, p/CIP, KAT13B | 66                     | Yes                                  |
| PRIP         | NCOA6, AIB3, ASC2, HOX1.1, HOX7, NRC, RAP250, TRBP | 67                     | Yes                                  |
| NCoR         | NCoR1                | 68                     | Yes                                  |
| SMRT         | NCoR2, SMRT, SMRT-τ  | 68                     | Yes                                  |
| IKKB         | IKKB, IKK-β, IKK2    | Unknown                | Yes                                  |
| DAX1         | NROB1                | 69                     | Yes                                  |
| SHP          | NROB2                | 70                     | Yes                                  |
| RIP140       | NRI1                 | 71                     | Yes                                  |
| GCN5         | GCN5L2, KAT2A, PCAF-b | Unknown                | No                                   |
| TRAP220      | MED1, CRSP1, CRSP200, DRIP205, DRIP230, PBP, PPARBP, PPARGBP, RB18A, TRIP2 | 72                     | Yes                                  |
| PGC1α        | PPARC1α, LEM6        | 38                     | No                                   |
| TRIP3        | ZNHIT3               | Unknown                | Yes                                  |
| TRIP4        | ASC-1                | Unknown                | No                                   |
| TRIP5        | KIF11, EG5, HKSP, KNSL1 | Unknown                | No                                   |
| TRIP8        | JMJD1C               | Unknown                | Yes                                  |

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To analyze the kinetics of the interactions, a peptide array was generated containing different amounts of TRAP220LLXXLLLL6942 peptide (Fig. 1d, SYPRO Ruby staining of a 2-fold dilution series). Dissociation constants (Kd) for the unliganded (DMSO) and liganded (rosiglitazone) form of GST-PPARγ-LBD were determined (Fig. 1d, upper panel) as was the ratio of Vmax of unliganded and liganded GST-PPARγ-LBD (modulation index (MI); Fig. 1d, lower panel) at each peptide concentration (see supplemental Fig. 2 for further explanation of $K_d$ and MI). Curves were obtained with high quality fits (global $R^2 > 0.994$) with $K_d$ values of 3.8 (log $\mu M$, no ligand) and 3.2 (log $\mu M$, rosiglitazone). The ratio of these $K_d$ values represents the drug efficacy for this interaction (see supplemental Fig. 2), which in this case means that a saturating concentration of rosiglitazone induces a GST-PPARγ-LBD conformation that results in a ~4-fold ($10^{0.9, 3.2}$-fold) increased affinity for the TRAP220LLXXLLLL694 motif. These findings illustrate that at peptide concentrations higher than 0.25 mm clearly detectable interactions with NR proteins ($V_{max}$) as well as robust ligand effects (MI) could be observed. Therefore all subsequent assays were performed with peptide concentrations of 1 mM.

To investigate whether this array allows interaction profiling of agonist- and antagonist-occupied NRs, we incubated the GST-tagged LBD of PPARγ with rosiglitazone (31) or GW9662 (32), respectively. In both cases binding to coregulator-derived peptides was measured using the PamChip-96 and Pamstation-96. Importantly the PPARγ-coregulator peptide interactions observed upon agonist treatment confirmed previous protein-protein interaction studies (Table 1): rosiglitazone specifically induced binding to the LXXLL motifs derived from the coactivators CBP, p300, SRC1, TIF2, SRC3, PRIP, IKBB, DAX1, SHP, RIP140, and TRAP220, whereas interactions with the corepressor motifs NCoRLLXXLLLL2263 and SMRTLXXXXXXL2342 were reduced compared with vehicle treatment (Fig. 2, a and b, and supplemental Fig. 3). In contrast to rosiglitazone treatment, the antagonist GW9662 failed to induce binding to any of the coactivator peptides and even slightly reduced several ligand-independent interactions while preserving the interactions with the NCoR and SMRT corepressor peptides (Fig. 2, a and b).

To characterize the binding properties of each spotted peptide on the array in more detail, dose-response curves were generated using increasing concentrations of rosiglitazone and GW9662 (2 × 10$^{-11}$–2 × 10$^{-4}$ M) (Fig. 2, c and d, and supplemental Fig. 3). For each peptide curve fitting was applied to determine EC50 and the difference between minimum and maximum binding (induction; supplemental Fig. 2), whereas the accompanying $R^2$ was used as quality measure for the fit. In the case of rosiglitazone, 30 of the total 48 peptide binding curves showed $R^2$ values of >0.7 (see supplemental Fig. 4 and supplemental Table 2). Within this group, EC50 values varied from $6 \times 10^{-7}$ M (TIF2LXXLLLL990) to $4 \times 10^{-8}$ M (CBPXXLLLL70). In the case of GW9662 only six peptides showed $R^2$ values of >0.7 (see supplemental Table 2) with most of the peptides showing the opposite effect of that obtained with rosiglitazone, reflecting the agonistic and antagonistic activity of rosiglitazone and GW9662, respectively. In conclusion, the peptide microarray approach allows dynamic studies on the interactions between a given NR and 48 coregulator motifs in a single experimental run.

The SPPARγM Telmisartan Induces a Specific PPARγ-Coregulator Interaction Profile—SPPARγMs are ligands that show partial modulatory effects on PPARγ activity compared with traditionally fully agonistic TZDs like rosiglitazone or pioglitazone (17). To establish a broad SPPARγM-induced coregulator interaction profile, PPARγ was incubated with telmisartan, an angiotensin receptor blocking drug with SPPARγM characteristics (33). NR-coregulator interactions were analyzed with PamChips containing 53 peptides. Using telmisartan and rosiglitazone concentrations ranging from 10$^{-9}$ to 10$^{-4}$ M, dose-response curves were generated.

A large number of peptides followed the binding pattern of rosiglitazone-treated GST-PPARγ-LBD only with reduced induction and/or increased EC50 values (Fig. 3, a and b, and supplemental Fig. 5). For instance, in the case of the PRIPLXXLLL1491 peptide, telmisartan showed an induction similar to that of rosiglitazone but with an increased EC50 (Fig. 3a). In the case of peptide PGClXXLLLL343, induction was reduced, and EC50 increased (Fig. 3, a and b). Furthermore in the case of the P300LXXLL8, and TRAP220LXXLL645 peptides, telmisartan failed to induce binding as only a minimal induction value was observed, whereas rosiglitazone treatment resulted in increased interactions (Fig. 3, a and b). The rosiglitazone-induced interaction between GST-PPARγ-LBD and the four SRC1 peptides on the array (SRC1LXXLL633, SRC1LXXLL900, SRC1LXXLL749, and SRC1LXXLL1439) was more efficient and potent compared with the effect of telmisartan (Fig. 3b). To study this interaction by independent experimental means, we performed GST pulldown experiments. GST-SRC1 fusion protein harboring three LXXLL motifs (amino acids 570–780) was incubated with $[^35]$S-labeled PPARγ in the absence or presence of rosiglitazone or telmisartan ($10^{-8}$–10$^{-4}$ M). In accordance with the PamChip data, rosiglitazone treatment resulted in a profound interaction between PPARγ and SRC1, whereas the effect of telmisartan was more modest (Fig. 3c). In conclusion, our data confirm and expand the qualification of telmisartan as a bona fide SPPARγM.

PPARγ Mutants Display Altered Coregulator Interaction Profiles—To investigate the effects of PPARγ mutations on the coregulator interaction profile, we used the naturally occurring R425C mutant and the artificial L468A/E471A mutant. The FPLD3-associated R425C mutant in which a conserved arginine at position 425 is changed into a cysteine (34) displays reduced ligand binding and ligand-mediated coactivator

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2 The position number refers to the first Leu of the peptide motif in the full-length protein.
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**Fig. 1.** Nuclear receptor-coregulator interaction profiling. 

**a.** PamChip design. The PamChip-96 consists of 96 identical arrays, each array consists of 48–53 coregulator peptide spots, and each spot is immobilized on a porous metal oxide carrier. **b.** NR-coregulator interactions. Peptides derived from NR coregulators (red), containing either LXXLL or LXXXIII IL motifs, are covalently attached to the porous carrier material (light gray). GST-NR fusion proteins (blue) are pumped through the porous material together with a fluorescently labeled antibody against GST (green). Depending on the presence of ligand, the GST-NR protein will bind to coregulator-derived peptides, which can be detected by the fluorescent antibodies. Background fluorescence signal is reduced because unbound protein-antibody complexes are collected behind an optic barrier (dark gray), taking these out of the focal plane of the optical detection system. **c.** Time-dependent binding of GST-PPARγ-LBD to immobilized TRAP220_XXL604 motif visualized by fluorescently labeled anti-GST antibodies (lower panel). During incubation, images were acquired at cycles 21, 31, 61, 81, and 101, and binding was quantified. Cycle number was plotted against signal, a
recruitment (23). As a result, the transcriptional activity of this mutant is reduced (Fig. 4a), ultimately leading to impaired capacity to induce adipocyte differentiation (23). The artificial L468A/E471A double mutant contains two alanine residues in helix 12 of the LBD instead of the highly conserved hydrophobic leucine and charged glutamic acid residues. This mutant still binds ligand, but coactivator binding is ablated, whereas corepressors are recruited more avidly in the absence of ligand (22). In agreement with earlier reports (22, 23), the ability of the PPARγL468A/E471A to activate a 3xPPRE-tk-Luc reporter was completely abolished (Fig. 4a).

We then subjected bacterially expressed and purified wild type and mutant (R425C and L468A/E471A; Fig. 4b) GST-PPARγ-LBD proteins to peptide microarray analysis in the absence or presence of rosiglitazone. Dose-response curves were determined for all coregulator-derived peptides. Depending on the quality of the curve fit ($R^2 > 0.5$) and the overlap of 95% confidential intervals of top and bottom values, potency (EC50) and induction values were calculated (Fig. 4c). Dose-response curves ($R^2 > 0.5$) could be obtained for most peptides. However, mutation of Arg-425 dramatically altered the binding characteristics of the PPARγ protein because in this case only five peptides showed dose-response curves with $R^2 > 0.5$ (SRC1LXXXL1435, DAX1LXXXL1465, NRIP1LXXL185C1775, PGC1αLXXXL144motif1), and PGC1αLXXXL144motif2) but with minimal induction values (Fig. 4, c and d). This profound effect on the overall interaction profile is in agreement with the reduced ligand binding affinity of the R425C mutant (23). The L468A/E471A mutant protein showed strongly reduced coactivator recruitment upon ligand stimulation with no peptide displaying significant ligand-induced interaction. Ligand-independent binding to LXXXIXXXL repressor motifs, however, was stronger than in the case of wild type PPARγ (Fig. 4, c and d), which is in line with the stronger recruitment of corepressors by this mutant (22). Taken together, these data indicate that naturally occurring and artificial PPARγ mutants display distinct coregulator interaction profiles in line with their functional characterization in in vitro and cell-based assays.

**PPAR Family Members Show Distinct Coregulator Binding Profiles**—The PPAR isotypes α, β/δ, and γ are, especially in their LBDs, highly homologous (>62% identical and >80% synonymous). These three NRs nevertheless regulate specific sets of target genes (35, 36), which may be due to differences in coregulator interactions. To investigate this possibility we stimulated GST-LBD versions of PPARα, -β/δ, and -γ with increasing amounts of their respective synthetic ligands, WY14643, GW501516, and rosiglitazone, and performed peptide microarray analyses. As depicted in Fig. 5a, the LBDs of PPARα, PPARβ/δ, and PPARγ, although highly homologous, differ to such an extent that distinct coregulator interaction profiles were observed. Clear differences were observed in ligand-independent binding, which was more pronounced for PPARγ compared with PPARα and PPARβ/δ (Fig. 5a). PPARβ/δ showed almost no ligand-independent binding, hence the pronounced effect of ligand on the overall binding profile (Fig. 5a). In addition, a number of peptides displayed isotype-selective interactions upon ligand treatment, including the PGC1αLXXXL144 (PPARβ/δ; please note that PPARα displayed inconsistent interaction patterns with the two PGC1α peptides (supplemental Fig. 7)), IKBBBBLXXLL289 (PPARβ/δ and -γ), and NCOR1LXXXLXXX2283 and SMRTLXXXLXXX2342 (PPARα and -γ) peptides (Fig. 5b and supplemental Fig. 7). In contrast, a second set of peptides showed increased binding upon ligand treatment for all three proteins, including the peptides CBPXXXXLXXX70, P300LXXXLXXX1, SRC1LXXXL1435, and PRIPLXXXLXXX47 (Fig. 5b and supplemental Fig. 7). As a control we incubated GST-PPARα-LBD with the PPARβ/δ ligand WY14643 and GST-PPARβ/δ-LBD with the PPARα ligand GW501516. Under these conditions, no ligand-dependent interactions were observed in any of the experimental settings (supplemental Fig. 8).

As depicted in Fig. 5b, a striking PPAR isotype difference was observed with respect to the interactions with the two PGC1α peptides (which differ only in length; see supplemental Table 1): PPARβ/δ displayed clear ligand-dependent binding, whereas the interactions with PPARγ were ligand-independent (Fig. 5b). Because NR-coregulator peptide interactions may be influenced by the concentration of spotted peptide (supplemental Fig. 2), we analyzed the interaction between the PPARβ/δ and -γ isotypes and the PGC1αLXXXL144 peptide using an array containing different peptide concentrations (Fig. 5c). PPARγ displayed only modest ligand-dependent binding, which decreased with increasing peptide concentration, PPARβ/δ, however, displayed considerable ligand effects even at the highest peptide concentration (Fig. 5c), indicating that the difference between PPARβ/δ and -γ observed in the standard assay (Fig. 5b; 1 mM peptide) is independent of peptide concentration. GST pulldown assays were performed to verify these findings (Fig. 5d). Indeed ligand treatment clearly increased the interaction between PGC1α and PPARβ/δ as observed earlier (26, 37). The interaction between PGC1α and PPARγ was highly ligand-independent with no ligand-induced binding, which was also reported before (38). These data
FIG. 2. Coregulator peptide binding patterns of GST-PPARγ-LBD treated with agonist rosiglitazone and antagonist GW9662. a, ligand effects for GST-PPARγ-LBD binding to the corepressor motif NCOR1LXXII2263 and the coactivator motif TRAP220LXXLL645. PamChip arrays were incubated with GST-PPARγ-LBD protein in the presence of DMSO (control), rosiglitazone (10 μM), or GW9662 (10 μM). The corepressor motif
therefore support the isotype-specific interactions observed in the peptide microarray experiments by independent experimental means.

TRIP3 Regulates PPARγ-mediated Adipocyte Differentiation—As indicated in Table I, TRIP3 had not been implicated in PPARγ signaling before. The 155-amino acid TRIP3 pro-
**Fig. 4.** Effects of mutations in PPARγ on coregulator interactions. 

*a*, transcriptional activation by PPARγL468A/E471A and PPARγR425C.

U2OS cells were transfected with expression vector encoding PPARγ2 wild type (wt), PPARγL468A/E471A, or PPARγR425C and a 3xPPRE-tk-Luc reporter. Activation of the luciferase reporter, in the absence or presence of 1 μM rosiglitazone, is expressed as -fold induction over that with
tein harbors a HIT type 3 zinc finger and a well conserved LXX LL motif (Fig. 6a) and is widely expressed (39, 40). TRIP3 has been shown to interact with multiple NRs: the protein was originally found to interact with thyroid hormone receptor β only in the presence of thyroid hormone (39). It also showed a ligand-dependent interaction with 9-cis-retinoic acid receptor α but did not interact with the glucocorticoid receptor under any condition (39). Subsequent studies showed an interaction with the orphan receptor hepatocyte nuclear factor-4α (40). The interaction profile showed a significant ligand-dependent interaction between the TRIP3 peptide and PPARγ (Figs. 4d and 6b; see also Figs. 2b and 5b and supplemental Figs. 3, 5, 6, and 7) that was abolished in the R425C and L468A/E471A mutants (Fig. 4d). To test the full-length TRIP3 protein for its ability to interact with PPARγ, we performed GST pulldown experiments in the presence or absence of rosiglitazone. A rosiglitazone-dependent interaction between full-length TRIP3 and GST-PPARγ-LBD was detected (Fig. 6c, upper panel). Importantly mutation of the LXXLL motif (TRIP3c) completely abolished this interaction (Fig. 6c, lower panel). Because of the key role of PPARγ in adipogenesis, we next investigated whether TRIP3 would also play a role in this process. As a first experiment, protein expression of TRIP3 was studied during the differentiation of 3T3-L1 cells into adipocytes (Fig. 6d). Induction of differentiation, as monitored by the expression of the differentiation marker FABP4, resulted in increased expression of the TRIP3 protein after 2–3 days. To address the relevance of TRIP3 in adipogenesis, the expression of this protein was reduced using RNA interference-mediated knockdown. Using a pool of siRNA oligonucleotides, TRIP3 expression was efficiently reduced in 3T3-L1 cells (Fig. 6e). Knockdown of TRIP3 resulted in a partial inhibition of adipocyte formation as illustrated by staining of triglycerides with oil red O at day 6 of differentiation (Fig. 6e). To confirm this reduction in adipocyte differentiation independently, protein expression levels of FABP4 and PPARγ were determined. Although the FABP4 and PPARγ proteins were clearly induced upon differentiation, treatment of cells with TRIP3 siRNA oligonucleotides blunted this response (Fig. 6e). Knockdown of PPARγ itself resulted in a complete inhibition of adipogenesis (Fig. 6e). These results therefore implicate the TRIP3 protein in PPARγ-mediated adipogenesis and indicate that novel, biologically relevant interactions can be identified based on the NR-coregulator interaction profiling method used here.

**DISCUSSION**

For NR-mediated gene transcription to occur, these proteins need to work in concert with transcriptional coregulators, including corepressors and coactivators. The specific set of NR coregulators in complex with an NR is determined by the cellular context, the type of NR, and the type of ligand. Here we used an in vitro microarray approach to functionally characterize ligand-induced NR-coregulator recruitment based on interactions between NRs and peptides derived from known NR coregulators.

A number of peptide-based methods have been described in which NR-coregulator interaction profiles can be generated, including homogeneous time-resolved fluorescence resonance energy transfer (41–44) and bead-based multiplexed measurements coupled to flow cytometry (45, 46). In addition, phage display library screening has been used to identify artificial interacting peptides (46–50). Methods in which full-length coregulator proteins rather than LXXLL/LXXXLXXXL peptides were used include high throughput GST pulldown assays (51), two-hybrid-based methods (52), and protein microarrays in which either NR or coregulators were immobilized (53). The peptide microarray approach we used has several advantages over these methods. First, it combines sensitive high throughput interaction profiling with real time kinetics, enhancing the reliability of the interaction data (Vₗ values: Fig. 1 and supplemental Fig. 1). Second, the peptide microarray approach avoids time-consuming cloning procedures. Third, only small amounts of NR protein (<10 ng of protein/array) are required with a relatively low degree of purity (data not shown).

An obvious application of high throughput NR-coregulator interaction profiling is the initial screening of novel NR (ant)agonists that may have clinical applications. Although NRs may be regarded as natural drug targets, NR-based drugs are often associated with (tissue-specific) adverse side effects. For example, synthetic PPARγ ligands of the T2D class, which are used in the treatment of insulin resistance associated with type 2 diabetes, have been associated with serious side effects like undesired weight gain, fluid empty vector (pCDNA3) in the absence of ligand after normalization for Renilla luciferase activity. Results are averages of at least three independent experiments assayed in duplicate ± S.E. b, protein isolation of GST-PPARγ-LBD, GST-PPARγ-LBDL468A/E471A, and GST-PPARγ-LBD_{R425C}. GST-PPARγ-LBD fusion proteins were bacterially expressed and purified. SDS-PAGE followed by Coomassie Brilliant Blue staining was performed to quantify protein amounts using BSA standards. c, coregulator binding profiles for GST-PPARγ-LBD wild type and mutants. PamChip arrays were used to analyze the ligand-dependent coregulator binding of GST-PPARγ-LBD, GST-PPARγ-LBD_{R425C}, and GST-PPARγ-LBD_{L468A/E471A}. Dose-response curves were determined upon rosiglitazone stimulation. EC₅₀ values and induction values were determined as described in Fig. 3b. d, selected examples of differences in corepressor release and coactivator recruitment between GST-PPARγ-LBD, GST-PPARγ-LBD_{R425C}, and GST-PPARγ-LBD_{L468A/E471A}. The different GST-PPARγ-LBD fusion proteins were treated with increasing concentrations of rosiglitazone, and coregulator binding was analyzed with PamChip arrays. Dose-response curves for the coregulator peptides NCOR_{LXXL2263}, SRC1_{LXXL1435}, TRIP3_{LXXL101}, and TRAP220_{LXXL604} are depicted. A.U., arbitrary units; WT, wild type. Error bars indicate standard errors (S.E.).
FIG. 5. Coregulator binding characteristics of PPAR isotypes. a, coregulator peptide binding patterns for PPARα, PPARβ/δ, and PPARγ in the presence (+) or absence (−) of ligand. PPARα, PPARβ/δ, and PPARγ were treated with 10 μM WY14643, 440 nM GW501516, and 10 μM rosiglitazone, respectively. DMSO was used for control experiments. The motifs PGC1αLLXXLL144 (light gray circle) and PGC1αLLXXLL144 (dark gray circle) are highlighted. b, coregulator peptide inductions for WY14643 (PPARα), GW501516 (PPARβ), and rosiglitazone (PPARγ). For each dose-response curve inductions were calculated according to the conditions described in Fig. 3b. For each PPAR isotype efficacies were normalized to the highest induction value obtained in the experiment. All dose-response curves were performed in duplicate. c, the effect of increasing amounts of PGC1αLLXXLL144 peptide on the ligand-induced interaction of PPARβ/δ and PPARγ with PGC1αLLXXLL144. Increasing amounts of PGC1αLLXXLL144 were spotted on a PamChip array, and at each peptide concentration Vₐᵦ values were determined either in the presence or absence of ligand. d, in vitro interaction between GST-PGC1α and PPARβ/δ or PPARγ. GST pulldown experiments were performed to analyze PGC1α binding by PPARβ/δ or PPARγ upon ligand stimulation. In vitro translated PPARβ/δ and PPARγ were treated with 440 nm GW501516 and 10 μM rosiglitazone, respectively, and subjected to a pulldown experiment with GST-PGC1α. Experiments were performed with DMSO as control. A.U., arbitrary units.
retention, peripheral edema, and potential increased risk of cardiac failure. These effects may be due to the use of high doses of full PPARγ agonists (14, 15). Activation in moderation through selective NR modulators (SNRMs), which elicit a more restricted, tissue-specific transcriptional response, may therefore be a more sensible approach (16, 17). The validity of the SNRM concept is clearly underscored by tamoxifen, which is widely used for the treatment and prevention of breast cancer: this drug functions as an estrogen receptor agonist in some tissues (e.g. bone) but as an agonist in others (e.g. breast) (54, 55). Besides SNRMs for PPARγ and estrogen receptor, such compounds are being...
developed by pharmaceutical companies for the progesterone receptor, androgen receptor, and glucocorticoid receptor (56). NR-coregulator interaction profiling allows characterization of compounds, including SNRMs, as illustrated by the interaction profile induced by the SPPARγM telmisartan (Fig. 3). Together with experiments assessing the selectivity of a compound for a given NR (Fig. 5 and supplemental Fig. 8), such analyses could aid the selection of effective compounds in the early stages of drug development to help minimize the costs of expensive gene expression analyses and animal studies.

Another interesting application of NR-coregulator interaction profiling is the functional characterization of NR mutants. A number of diseases are associated with mutations in NR genes, frequently affecting the LBD (57, 58). In some cases, the functional defect can be (partially) rescued by specific ligands as exemplified by the effects of synthetic tyrosine-based agonists on the FPLD3-associated PPARγ V318M and P495L mutants (59). Characterization of NR mutants using NR-coregulator interaction profiling can therefore provide the rationale for therapy in such cases.

Several novel ligand-dependent interactions were observed in our interaction profiling experiments. Previously unknown interactions (Table I) include those of PPARγ with IKKB, GCN5, TRIP3, and TRIP8 (Fig. 2 and supplemental Figs. 3, 5, 6, and 7). Our data further implicate TRIP3 in PPARγ-mediated adipocyte differentiation. In yeast, TRIP3 lacks intrinsic transcriptional activity when tethered to DNA (39), suggesting that its function as a transcriptional coregulator depends on other mammalian proteins. Interestingly the TRIP3 protein harbors a HIT type zinc finger, named after the yeast HIT1 protein, which is mainly found in nuclear proteins involved in gene regulation and chromatin remodeling (60). Recent studies on the closely related ZNHIT2 protein revealed that this type of zinc finger is not involved in DNA binding but probably serves as a protein-protein interaction surface (61). Future studies are therefore required to reveal the exact role of TRIP3 and its HIT type zinc finger in PPARγ-mediated transcription processes.

Although best known for their application in enzymatic studies on kinases and proteases (21), our studies indicate that peptide microarrays may have wider applications in protein-protein interaction studies. Of particular interest in this respect are docking interactions of kinases and phosphatases with their substrates, which critically depend on short peptide motifs within these substrate proteins (62). In addition, this technology may be extended toward the interactions between N-terminal histone tails and their binding partners in which the interactions are dictated by specific posttranslational modifications (63). Because NRs, kinases/phosphatases, and histone-interacting proteins like lysine and arginine methylases are involved in many diseases and often qualify as potential drug targets, peptide microarray platforms may present an important generic tool in drug development.

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