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Binding and Stability Determinants of the PPARγ Nuclear Receptor−Coactivator Interface As Revealed by Shotgun Alanine Scanning and in Vivo Selection

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Abstract: We modified an existing selection for protein−protein interactions based on the fragment complementation of the enzyme DHFR. Using shotgun alanine scanning in conjunction with this selection, we analyzed the interaction of the nuclear receptor PPARγ with two peptides derived from nuclear receptor coactivators SRC1 and TRAP220. A large binding epitope stretching between and including the charge clamp residues K301 and E471 of PPARγ was identified as necessary for PPARγ−coactivator interaction. To decouple protein stability from the propensity to form a receptor−coactivator interface, libraries of PPARγ variants generated by shotgun scanning were further processed using a high-throughput screen measuring their in vivo stabilities. Our findings demonstrate that many of the residues that make up the binding epitope of PPARγ are also crucial for the stability of the PPARγ.

Introduction

Nuclear receptors (NRs) are a superfamily of transcription factors that regulate a wide variety of genetic programs, including sexual development and adipogenesis. NRs typically contain a DNA-binding domain and a ligand-binding domain (LBD).[1] The LBDs of many nuclear receptors bind lipophilic small molecules, including steroid hormones, and exhibit a high degree of structural similarity. In the absence of ligand, nuclear receptors interact with corepressors, proteins that inhibit transcriptional activation. Upon ligand binding, the receptors undergo a conformational change that causes corepressor disassociation and enables nuclear receptor coactivators to bind.[2] Coactivator binding ultimately results in the recruitment and assembly of the transcriptional machinery.

The C-terminal helix (helix 12) of a nuclear receptor LBD is thought to exhibit conformational mobility in the unliganded state. Ligand binding favors a state in which helix 12 is well-packed against the rest of the receptor.[3] This conformational change creates, on the surface of the receptor, a highly conserved hydrophobic cleft known as Activation Function-2 (AF2). AF2 serves as a docking site for the highly conserved LXXLL motif present in nuclear receptor interacting domains (NRIDs) of coactivator proteins. NRIDs are natively disordered regions of coactivator proteins that undergo a structural transition upon AF2 binding. NRIDs form a short three-turn helix upon interaction with AF2,[4] and biochemical studies have shown this short motif of approximately 10 residues to be sufficient for binding.[5,6] Accordingly, NRIDs serve as simple, peptidic modules that can mimic the interaction between NRs and full coactivator proteins.

The peroxisome proliferative activated receptor, gamma (PPARγ), is a nuclear receptor that is necessary for adipogenesis and is also the target of the thiazolidinone class of antidiabetic drugs.[7] PPARγ has been shown to stimulate transcriptional activation upon binding to numerous LXXLL-containing coactivator proteins. Despite the high level of conservation of both the AF2 binding cleft of nuclear receptors as well as the LXXLL motif of the coactivators, the determinants of nuclear receptor−coactivator binding have not been studied in a manner that comprehensively probes all interface residues. Here we describe studies to illuminate the determinants of the interaction between PPARγ and LXXLL-containing coactivators. We modified a genetic selection that links cell survival to protein−protein interaction in order to enable the simultaneous evaluation of interactions between many PPARγ and coactivator peptide variants. Using this selection, we carried out several shotgun alanine scans of PPARγ−coactivator interfaces to reveal the functional determinants of this interaction. Finally, we integrated shotgun alanine scanning of PPARγ with a high-throughput screen for protein stability to further dissect the roles played by individual essential residues. Taken together, our findings identify determinants of PPARγ−coactivator interaction as well as determinants of PPARγ stability. In addition, these insights may facilitate the laboratory evolution of PPARγ and coactivator

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variants that are not subject to crosstalk with endogenous nuclear receptor/coactivator pairs.

Materials and Methods

Molecular Biology Reagents. All enzymes were purchased from New England Biolabs, except for *Pfu* DNA polymerase (Stratagene). Sequencing was performed by the Harvard MCB Sequencing Core Facility and by ACGT, Inc. The anti-GFP antibody was purchased from Clontech.

Mutagenesis. Unless otherwise noted, mutagenesis used the protocol of Miyawaki and co-workers.[8] 5′-Phosphorylated oligonucleotides (Integrated DNA Technologies) containing degenerate shotgun alanine scanning codons described by Weiss et al.[9] were used to mutagenize a template plasmid that contains stop codons incorporated into the gene of interest (PPARγ, SRC1, or TRAP220) at each region to be mutated or scanned. Typically, 500 ng of the phosphorylated primer containing the mutagenic codons and wild-type flanking regions was added to 200 ng of the template plasmid in 0.5× *Taq* ligase buffer, 0.5× *Pfu* polymerase buffer, 200 μM dNTPs, 40 U *Taq* ligase, and 5 U *Pfu* DNA polymerase. Thirty cycles of PCR were performed with an extension time of 2 min/kb. The product of the PCR was digested with *Dpn* I for 1 h, extracted with phenol/chloroform, and buffer-exchanged into pure water. The resulting DNA was transformed into electrocompetent DH10B cells (Invitrogen) and plated on large 2xYT/agar plates including the appropriate antibiotic (carbenicillin for the PPARγ scans, and chloramphenicol for the TRAP220 and SRC1 scans). The cells were harvested and the amplified plasmid DNA was isolated by standard methods. For all scans, library sizes were $\geq 5 \times 10^7$ transformants. DNA sequencing from randomly chosen clones indicated that libraries typically contained approximately 90% appropriately mutated sequences after this mutagenesis protocol.

DHFR* Fragment Complementation Plasmids. pBS-BAD-PPARc2H2 features the PPARγ ligand-binding domain fused to the N-terminus of DHFR*. pBS-BAD-PPAR is identical to pBS-BAD-PPARc2H2, except that it lacks the L28R mutation within the DHFR gene. pBC-BAD-SRC1 features a fusion of GST, a short peptide of SRC1 (residues 685−700), and the C-terminus of DHFR*. Both vectors feature the araBAD promoter from pBAD upstream of the protein fusions, allowing their induction with arabinose. pBS-BAD-PPARc2H2 contains the ColE1 origin and β-lactamase marker derived from pBluescript (Stratagene). pBC-BAD-SRC1 contains the ColE1 origin and the chloramphenicol resistance gene from pBC (Stratagene). Protein sequences of the fusion constructs are shown below.

PPARγ LBD-N-DHFR* (in pBS-BAD-PPARc2H2): MASEYPYDVPDYAESADLRALAKHLYDSYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEIIYTMLASLMNKDGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPLLQEIYKDLY**GTGGSGGSGGSGGNGGSGGPG***MISLIAALAVDRVIGMENAMPWNLPADRAWFKRNTLNKPVIMGRHTWESIGRPLPGRRNIILSSQPGTDDRVTWVKSVDEAIAACG*. The linker between the domains is in bold, and the N-terminus of DHFR* (residues 1−85) is italicized.

GST-SRC1-C-DHFR* (in pBC-BAD-SRC1): MASPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPK**SDGGSGGS**ERHKILHRLLQEGSPS**GTGGSGGSGGSGGSGGSGGP***GDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERR*. The linker residues are shown in bold, and the DHFR* C-terminus (residues 86−159) is italicized. The third NRID of SRC1 (residues 685-700) is underlined.

pBC-BAD-TRAP220 is identical to pBC-BAD-SRC1, except the SRC1 NRID in the latter is replaced by the TRAP220 NRID (residues 643−651) **PMLMNLLKD**.

Selection of the Trimethoprim-Resistant DHFR* Mutant. Codons corresponding to residues L28, F31, I50, and L54 of the *Escherichia coli* DHFR fragment of pBS-BAD-PPAR were mutated to degenerate NNS codons via the method of Kunkel.[10] The resulting library of pBS-BAD-PPAR mutants was co-transformed with pBC-BAD-TRAP220 into DH10B cells. Cells were plated on selection media containing 50 or 100 μg/mL trimethoprim (Sigma). The largest colonies surviving the selection were picked and sequenced.

Alanine Scanning Selections. Approximately 200 ng each of the two DHFR* complementation vectors were co-transformed into 100 μL of electrocompetent DH10B cells (Invitrogen). The cells were rescued with 1 mL of SOC medium for 30 min at 37 °C, washed once with 1 mL of M9 minimal medium, and resuspended in M9. Aliquots of the cells were plated onto selection media containing various concentrations of trimethoprim (typically 20, 30, and 40 μg/mL) and grown at 37 °C for 36 h. Selection media consists of 1× M9 media, 0.5% glycerol, 0.8% casamino acids, 100 μg/mL carbenicillin, 40 μg/mL chloramphenicol, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 μg/mL thiamine, 0.02% arabinose, and 50 μM rosiglitazone (Apin Chemicals). Colonies were isolated from plates containing the highest level of trimethoprim that allowed growth, and the plasmid DNA in surviving colonies was isolated and sequenced following standard protocols.

Randomization of the LXXLL Motif. Selections were performed as in the alanine scanning selection described above, except an additional liquid selection step was performed prior to plating. Briefly, DH10B cells that had been co-transformed and washed with M9 were transferred to liquid selection media containing trimethoprim and grown until at stationary phase. Plasmid DNA from these selection cultures was isolated and then used to transform fresh DH10B cells before plating on selective media as described above.

GFP-Based Stability Screening. The plasmid used for GFP-based stability screening consists of pBAD-GFP,[11] with the PPARγ LBD (residues 207−477) cloned as a C-terminal fusion following a $(GGS)_3$ linker. Mutagenesis was performed as described above for the shotgun alanine scans used in the DHFR* complementation assay. The resulting libraries were transformed into DH10B and plated on LB/agar plates containing 100 μg/mL carbenicillin and 0.2% arabinose. A handheld UV lamp was used to illuminate the plates, and colonies were selected on the basis of visual inspection. Colonies that exhibited green fluorescence equivalent to that of the wild-type PPARγ/GFP fusion were isolated, and the corresponding plasmid DNA was sequenced using standard methods.

Results

Development of a Modified DHFR Fragment Complementation System for Selecting Protein−Protein Interactions. Phage display has been used to re-engineer protein interactions[12] and to determine binding epitopes using shotgun scanning methodologies.[9] Initial attempts to apply phage display to PPARγ, however, were unsuccessful. We turned instead to

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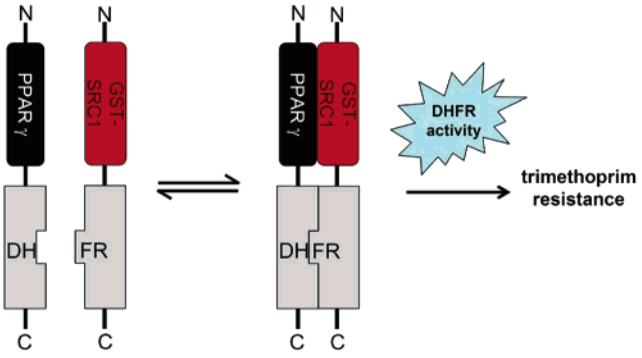

Figure 1. Overview of the DHFR fragment complementation assay[13,14] modified in this work.

dihydrofolate reductase (DHFR) fragment complementation,[13,14] a technique developed by Michnick and co-workers, in which each of two proteins being analyzed is translationally fused to two halves of a fragmented, and therefore nonfunctional, DHFR enzyme. If the two analyte proteins interact to form an intermolecular complex, the increased effective molarity of the DHFR fragments favors assembly of an active DHFR enzyme. If cells expressing these proteins are grown under conditions that require DHFR activity for survival, the resulting selection system could, in principle, link cell survival with the ability of two proteins to interact. The stringency of the selection could be controlled by using the DHFR inhibitor trimethoprim. Cells expressing interacting proteins that are able to reconstitute higher levels of DHFR should grow faster in the presence of trimethoprim and are able to grow at higher trimethoprim concentrations (Figure 1). Non-interacting proteins should be unable to reconstitute DHFR and should not survive in the presence of higher levels of trimethoprim.

We constructed two bacterial plasmids enabling the coexpression of (i) PPARγ fused to the N-terminal half of murine DHFR (mDHFR)$^{1-107}$, together with (ii) a fusion of glutathione-*S*-transferase (GST), TRAP220$^{643-651}$, and the C-terminal half of mDHFR$^{108-187}$ following previous reports.[13,14] TRAP220$^{643-651}$ is a coactivator peptide containing an LXXLL motif that forms a high-affinity complex with PPARγ. Although coexpression of these two fusion proteins should, in theory, reconstitute DHFR activity, we were unable to observe any growth advantage of *E. coli* containing these constructs compared with control cells not expressing the mDHFR fusions under selective conditions.

Control studies using a vector expressing mDHFR containing a $(Gly\text{-}Gly\text{-}Ser)_5$ loop inserted between residues 107 and 108 as a model of the reconstituted mDHFR also resulted in little growth advantage in the presence of trimethoprim and exhibited toxicity in *E. coli* DH10B and XL1-Blue strains (data not shown). We hypothesized that toxicity and poor expression of mDHFR may be hampering the selection, and therefore we replaced the mDHFR fragments with the homologous regions of the *E. coli* DHFR gene. This modification significantly improved the signal of the selection, allowing 50% of transformed *E. coli* cells to survive when plated on 5 μg/mL of the DHFR inhibitor trimethoprim, compared to an IC_{50} of 0.5 μg/mL of the control in which expression of the DHFR fusions was not induced.

To improve further the signal-to-background ratio of the fragment complementation system, we evolved mutant *E. coli* DHFR fragments that provide increased trimethoprim resistance. Toward this goal, we created libraries of *E. coli* DHFR using two methods. The first method used mutagenic dNTPs[15] during PCR to randomly mutagenize the DHFR portion of the DHFR^N fusion. The second method used a cassette mutagenesis strategy to specifically target DHFR residues L28, F31, I50, and L54, which likely contact trimethoprim on the basis of the structure of DHFR bound to the inhibitor methotrexate.[16] Libraries were transformed into *E. coli*, and colonies that survived on higher levels (50 or 100 μg/mL) of trimethoprim were isolated. All 20 sequenced surviving clones from the targeted library possessed the L28R mutation, while 14/18 clones selected from the random mutagenesis library possessed the same L28R mutation. Although this mutation is not known to impart trimethoprin resistance in *E. coli*, the analogous mutation of human DHFR has been reported to confer resistance to the DHFR inhibitor methotrexate.[17,18]

We found the L28R mutation to increase the IC_{50} of cells possessing a positive PPARγ−TRAP220$^{643-651}$ interaction from 5 to >100 μg/mL. We refer to this trimethoprin-resistant *E. coli* enzyme mutant below as DHFR*. The fragment complementation selection was then re-evaluated using DHFR* fragments fused to the partners of three protein−protein interaction pairs: (i) PPARγ−TRAP220$^{643-651}$ in the presence of the PPARγ ligand rosiglitazone, which confers a PPARγ−TRAP220$^{643-651}$ K_d of approximately 170 nM;[19] (ii) PPARγ−TRAP220$^{643-651}$ without rosiglitazone, which has an approximate K_d of 600 nM;[19] and (iii) PPARγ−TRAP220$^{643-651}$-(AXXAA), in which the three conserved leucines of the LXXLL motif have been mutated to alanine, which is expected to result in a K_d > 10 μM. In the presence of 60 μg/mL of trimethoprim, we observed a qualitative correlation between the affinity of the interacting partners among all three pairs and colony growth rates in the DHFR* fragment complementation system (Figure 2). On the basis of these results, we concluded that our modified DHFR* fragment complementation system is capable of selecting for interactions between PPARγ and potential coactivator partners corresponding to dissociation constants in the mid- to high-nanomolar range.

Control Library Selection Using the Nuclear Receptor Interaction Domain (NRID). A fusion analogous to TRAP-220$^{643-651}$−DHFR*^{C} was created using the nuclear receptor interaction domain (NRID) consisting of residues 686−700 from the coactivator SRC-1. SRC-1$^{686-700}$ contains the sequence ^{689}**IL**HR**LL**^{694} that has been shown to interact with the PPARγ with a dissociation constant of K_d = 31 nM.[19] Four residues of the NRID, including the three leucines that make up the canonical LXXLL motif, were randomized to create the library **XX**HR**XX** (theoretical library size of 160 000 coactivator variants). The DHFR* fragment complementation selection was used to select those coactivator variants that most strongly

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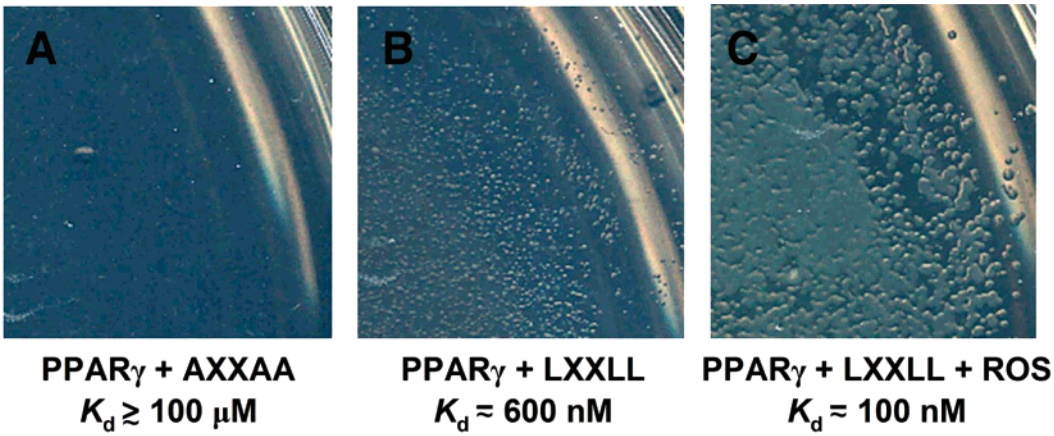

Figure 2. DHFR* fragment complementation control selections. (A) Plasmids expressing PPARγ−DHFR^{*N} and a non-interacting mutant TRAP220$^{643-651}$-Ala−DHFR*^{C} containing three Leu-to-Ala mutations within the LXXLL were co-transformed in *E. coli* and grown on selective media containing 60 μg/mL trimethoprim (see Materials and Methods for details). (B) Plasmids expressing PPARγ−DHFR^{*N} and TRAP220$^{643-651}$−DHFR*^{C} were co-transformed as in (A) and grown in the absence of rosiglitizone. (C) Identical to (B), but grown in the presence of 50 μM rosiglitizone. Expected K_d values for each PPARγ−coactivator interaction are shown. K_d values are estimates based on data from ref 13.

Figure 3. Sequence logo resulting from a coactivator peptide library selected for interaction with PPARγ. The third NRID of SRC1 (^{689}ILHRLL^{694}) was randomized to XXHRXX, where X represents any amino acid, and selected as described in the text. The height of each colored letter reflects the relative frequencies of amino acids among 22 sequenced surviving clones.

interacted with PPARγ. Among the surviving clones sequenced, 20 out of 22 (91%) contained the sequence **L**HR**LL**, while the remaining two clones contained the closely related sequence **L**HR**ML** (Figure 3). All clones were unique at the genetic level, indicating that they had been selected independently. These results demonstrate the ability of the DHFR* selection system to enrich for coactivator variants that possess known PPARγ affinity.

Shotgun Alanine Scan of PPARγ and the Coactivator. After developing and validating the modified protein−protein interaction selection, we sought to apply this approach to reveal the contributions of individual residues to the PPARγ−coactivator interface. Using the shotgun alanine scanning method of Weiss and co-workers,[9] degenerate codons were introduced into the PPARγ gene that allowed specified interface residues to be mutated to Ala or to remain wild-type (wt). Groups of seven PPARγ residues were mutated in two separate libraries; in total, 14 PPARγ residues were allowed to vary. Given the limited codon set used for alanine scanning, the theoretical diversity of the libraries was always less than 10^4 protein sequences. Library sizes for all alanine scans described below each exceeded 5×10^7 transformants, suggesting excellent coverage of all possible protein sequences. The resulting PPARγ libraries were subjected to the DHFR* selection system for interaction with the coactivator from the third NRID of SRC-1 (residues 686−700). Results of selections between SRC-1$^{686-700}$ and PPARγ are shown in Table 1 and in Figure 4.

The alanine scan results demonstrate that six residues located in the central portion of the interface (K301, F306, Q314, L318, K319, and I472), including the "charge clamp" residue K301, are absolutely intolerant to substitution with alanine. These residues therefore are likely crucial to the PPARγ−SRC1$^{686-700}$ interaction. E471, the opposing residue of the charge clamp, is known to be necessary for many NR−coactivator interactions. Even though this residue did not exhibit absolute conservation, it was still highly conserved (wt/Ala ratio after selection = 15.8) and therefore plays a significant role in the PPARγ−SRC1$^{686-700}$ interaction. The flanking residues of the interface (V293, L311, V315, and L468) contribute moderately to the interaction and possess wt/Ala ratios ranging from 3 to 7 (Table 1).

Table 1. Results of PPARγ Shotgun Alanine Scanning Selections

	PPARγ + SRC1					PPARγ + TRAP220				
PPARγ residue	wt	Ala			wt/Ala ratio	wt	Ala			wt/Ala ratio
V293	58V	14A			4.1	40V	18A			2.2
Q294	15Q	28A	25E	4P	0.5	6Q	29A	20E	3P	0.2
T297	35T	37A			0.9	26T	32A			0.8
K301	72K	0A			>72	58K	0A			>58
F306	72F	0A			>72					
L311	27L	8A	23V	12P	3.4					
Q314	72Q	0A			>72	47Q	0A			>47
V315	51V	16A			3.2	30V	17A			1.8
L318	61L	6A	0P	6V	>61	42L	0A	5V	0P	>42
K319	60K	0A	0E	7T	>60	47K	0A			>47
V322	44V	23A			1.9	36V	11A			3.3
L468	41L	6A	20V	0P	6.8	50L	0A	0V	0P	>50
E471	63E	4A			15.8	50E	0A			>50
I472	54I	0A	13V	0T	>54	42I	0A	8V	0T	>42

A similar shotgun scan of PPARγ was undertaken using the third NRID from TRAP220$^{643-651}$ as the coactivator sequence (Figure 5). The results are qualitatively similar to those seen for the interaction with SRC-1$^{686-700}$, although E471 and L468 are significantly more tolerant to alanine substitution in the PPARγ−SRC1$^{686-700}$ interaction compared with the PPARγ−TRAP220$^{643-651}$ interaction (Table 1 and Figure 4).

We then performed the complementary shotgun alanine scan by varying residues of the SRC1$^{686-700}$ coactivator peptide while using wild-type PPARγ in the protein interaction selection. Coactivator residues H687, I689, L690, H691, R692, L693, and L694 of SRC1 were allowed to vary between Ala and the wild-type residue, and the resulting library was selected using the

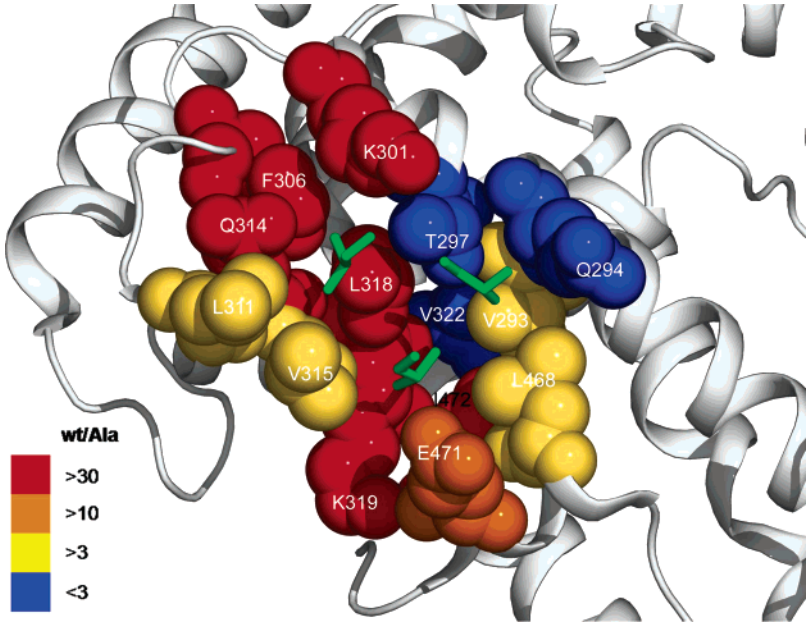

Figure 4. Shotgun alanine scan of PPARγ coupled with selection for interaction with coactivator SRC1 residues 685−700. Residues of PPARγ varied in the alanine scan are rendered as colored spheres. The side chains of the three leucines of the LXXLL motif of SRC1 are shown as green sticks.

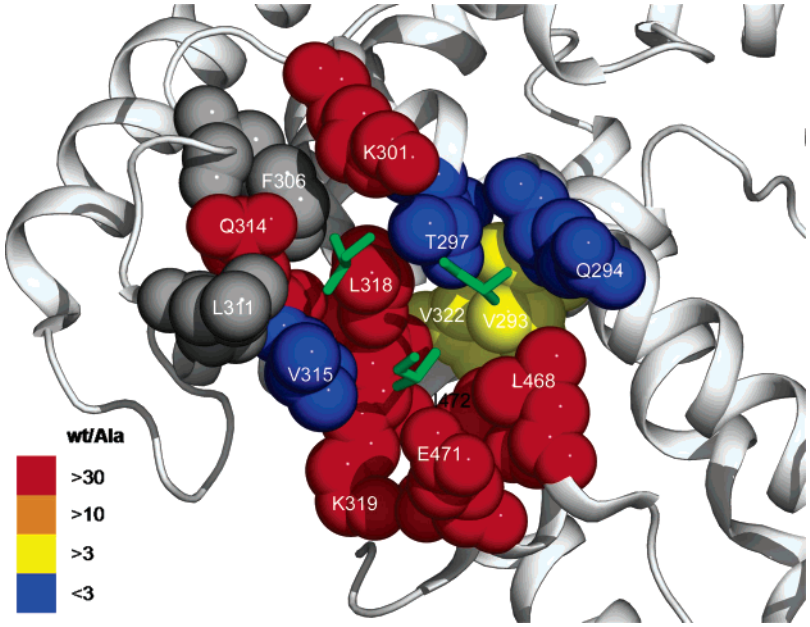

Figure 5. Shotgun alanine scan of PPARγ coupled with selection for interaction with coactivator TRAP220 residues 643−651. Residues of PPARγ varied in the alanine scan are rendered as colored spheres. The side chains of the three leucines of the LXXLL motif of SRC1 are shown as green sticks.

above method for interaction with PPARγ. The results of this coactivator shotgun alanine scan are shown in Figure 6. As expected, the L690, L693, and L694 residues that make up the canonical LXXLL motif show little or no tolerance to alanine substitution. H687 and I689, which bury substantial surface area upon complexation with PPARγ, also contribute significantly, albeit not as strongly, to the SRC1−PPARγ interaction.

PPARγ Stability. With the exception of the charge clamp residues K301 and E471, many of the residues that are highly conserved in the above alanine scans are largely buried, even in the absence of coactivator peptide. This observation raises the possibility that the invariance of these residues could arise not only from their participation in the PPARγ−coactivator interaction, but also from their role in the overall intracellular stability of PPARγ. To distinguish these two possibilities, we carried out an additional shotgun alanine scan of PPARγ coupled with a high-throughput intracellular screen for protein stability.[20]

We integrated shotgun alanine scanning with the previously validated use of GFP as an intracellular reporter of a protein's ability to fold into a stable form.[21] When fused with GFP, proteins that are unstable do not lead to fluorescence.[21] We hypothesized that the degree to which PPARγ−GFP fusions could be expressed as fluorescent proteins in *E. coli* would reflect the stability of PPARγ−N-DHFR* fusions used in the above fragment complementation selections.

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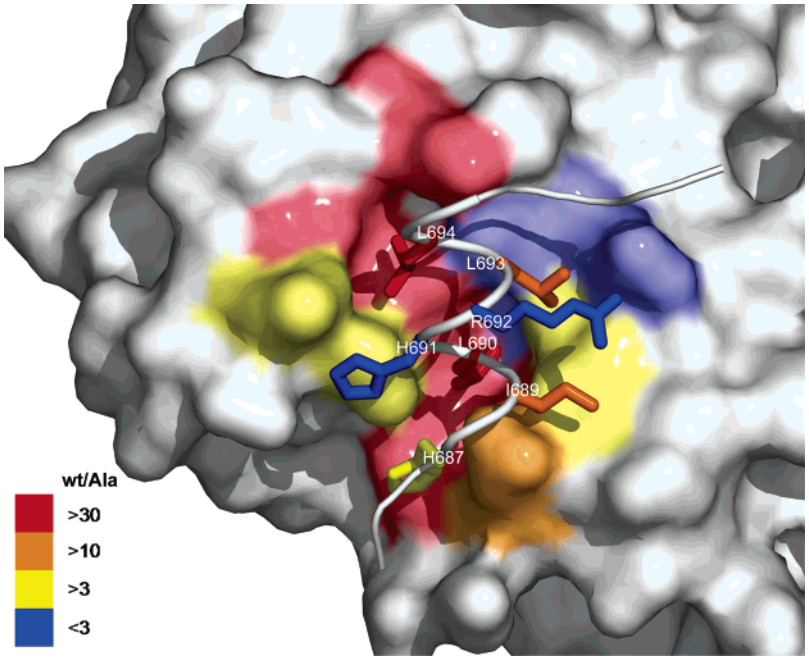

Figure 6. Shotgun alanine scan of SRC1 residues 687−694 (rendered as sticks) coupled with selection for interaction with PPARγ. The molecular surface of PPARγ is shown and colored as in Figure 4.

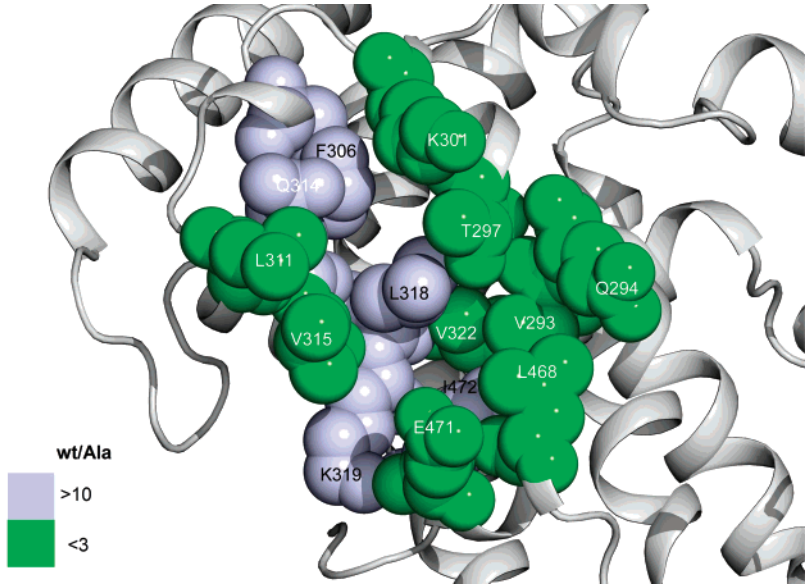

Figure 7. Shotgun alanine scan of PPARγ coupled with a GFP-based protein stability screen. Residues of PPARγ varied in the alanine scan are rendered as spheres. The ratios of wt/Ala residues among clones exhibiting green fluorescence are indicated by the coloring scheme shown above.

PPARγ was fused to the C-terminus of GFP, and shotgun libraries were generated as described above. Bacterial colonies that exhibited green fluorescence comparable to that of wild-type PPARγ fused with GFP were identified by visual inspection under UV light, and their PPARγ-encoding plasmids were sequenced (Figure 7). Few or no clones were discovered that contained alanine mutations for residues F306, Q314, L318, K319, and I472, suggesting that these five residues are necessary for the stability of PPARγ. The remaining nine interface residues (V293, Q294, T297, K301, L311, V315, V322, L468, and E471), however, demonstrated little preference for wild-type residues over alanine and, consequently, do not appear to play a major role in PPARγ stability.

To validate the results of the shotgun stability scan, we generated and individually characterized three PPARγ mutants fused to GFP on the basis of the above screening results. Two mutants identified in the screen as unstable, F306A and Q314A, when constructed and expressed individually, exhibited no fluorescence. In addition, Western blots revealed no stable expression of either the F306A or the Q314A mutant (Figure 8), consistent with their crucial role in PPARγ stability. Conversely, we also constructed the GFP fusion of the quadruple mutant V293A Q294A K301A L311A, containing alanine mutations at four residues which the GFP screen indicated play little role in PPARγ stability. Consistent with the ability of the GFP screen to identify stable and unstable PPARγ variants, this

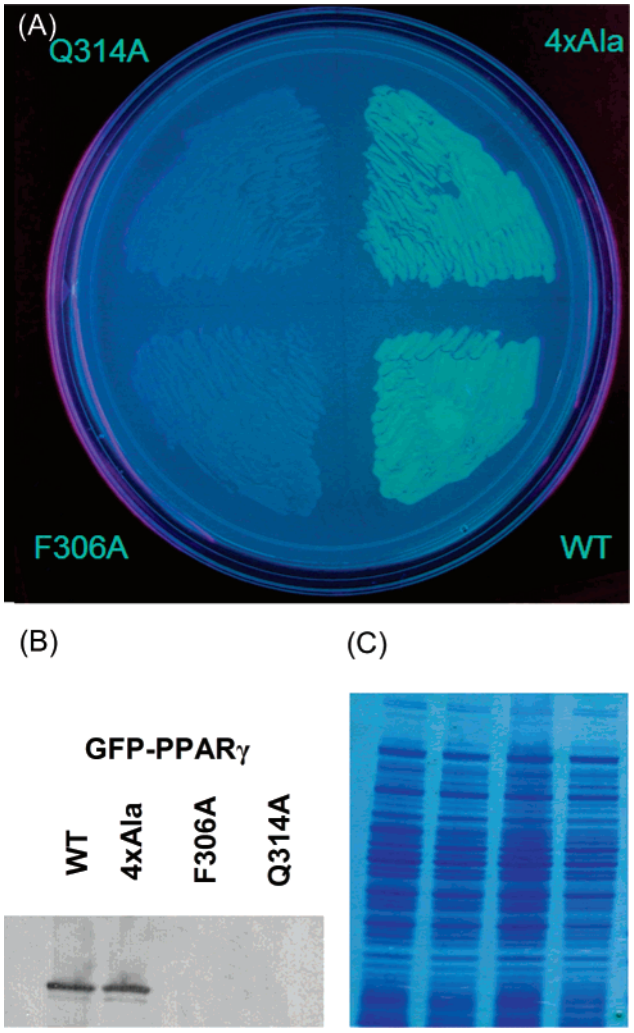

Figure 8. Mutations to the AF2 of PPARγ decrease stability of the PPARγ in *E. coli*. (A) *E. coli* expression fusions between GFP and PPARγ variants were grown on solid media and visualized under UV light. 4×Ala refers to the V293A Q294A K301A L311A quadruple mutant. (B) Western blot using anti-GFP antibodies of the GFP−PPARγ fusions expressed in (A). (C) Coomassie-stained gel of total protein in (B) is shown to reveal the amount of total protein in each lane.

quadruple mutant indeed exhibited no loss of fluorescence relative to GFP−wtPPARγ and no decrease in intracellular expression levels as judged by Western blot (Figure 8).

When compared with the results of the PPARγ-coactivator scan described above, these findings collectively suggest that PPARγ residues K301, L468, and E471 and coactivator residues I689, L690, L693, and L694 play crucial roles in forming a complementary PPARγ−coactivator interface. Our data also demonstrate that PPARγ positions F306, Q314, L318, K319, and I472 are required for the intracellular stability of the receptor and may also play a role in interactions with coactivator peptides.

Discussion

Shotgun scanning methods coupled with high-throughput functional assays such as phage display affinity selection or, in this case, intracellular DHFR* fragment complementation selection can rapidly identify key epitopes in protein−protein interactions. Traditional alanine scanning methods report directly on binding energetics between two binding partners and therefore typically require the purification and assay of multiple different alanine-containing mutant proteins. For the rapid determination of binding epitopes, however, shotgun methodologies may be preferable due to their high efficiency and simplicity. In addition, protein−protein interaction selections using DHFR* complementation or GFP-based stability screens take place intracellularly in contexts that may be more relevant to the study or future application of the proteins of interest. Like phage display shotgun scans, these experiments indirectly query binding interactions by rapidly assaying thousands of variants for their ability to function. For this reason, we interpret our results in terms of functional significance rather than in energetic terms; this distinction is crucial in our analysis of PPARγ−coactivator interactions.

Table 2. Results of the SRC1 Shotgun Alanine Scanning Selections

	SRC1 + PPARγ				
SRC1 residue	wt	Ala			wt/Ala ratio
H687	34H	4A	3P	4D	8.5
I689	29I	2A	0V	13T	14.5
L690	44L	0A	0P	1V	>44
H691	9H	36A	0P	0D	0.3
R692	8R	14A	0P	23G	0.6
L693	42L	2A	0P	1V	24
L694	35L	0A	0P	10V	>35

Pioneering work by Wells and co-workers on human growth hormone and its receptor led to the "hot spot" model of protein−protein interaction, whereby a small number of residues making up the interface between two transiently interacting proteins contribute a large percentage of the binding affinity. For example, two tryptophan residues of the human growth hormone receptor account for approximately 70% of the total binding energy between the receptor and growth hormone.[22] Although we find a hot spot of clustered residues (K301, F306, Q314, L318, K319, I468, and E471) on the PPARγ surface that are functionally necessary for PPARγ's interaction with coactivator NRIDs, we were surprised by the number of residues involved. Six of the 14 residues of the of the PPARγ that were included in the scans were found to be completely intolerant to substitution by alanine (Figure 4 and Table 1) and are thus functionally required for the interaction with NRIDs. Many of these key residues are also relatively intolerant to conservative mutations that are introduced as a byproduct of shotgun alanine scanning.

In the above studies, we mutated residues of the PPARγ that are in close contact with residues of the SRC1 coactivator peptide.[19] We did not expect that mutation of residues contributing to the molecular surface of PPARγ would have a major effect on protein stability or expression.[23] Surprisingly, our results indicate that F306, Q314, L318, K319, and I472, which collectively form much of the functional binding epitope as identified in the fragment complementation selection, are also absolutely necessary for the intracellular stability of PPARγ and are relatively intolerant to even conservative L318V and I472V mutations, as revealed by the GFP-based screen and Western blots (Figures 7 and Table 3). A salient feature of PPARγ−coactivator interactions may be the overlap of residues involved in the proper folding and stability of PPARγ with residues that

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Table 3. Results of the GFP-Based PPARγ Shotgun Alanine Scanning Stability Screen

PPARγ residue	wt	Ala			wt/Ala ratio
V293	51V	32A			1.6
Q294	20Q	20A	5P	22E	1.0
T297	39T	36A			0.9
K301	33K	21A	11T	13E	1.6
F306	56F	0A	0P	0V	>56
L311	13L	18A	20P	23V	0.7
Q314	53Q	2A			26.5
V315	45V	31A			1.5
L318	64L	3A	0P	9V	21.3
K319	41K	2A	3T	25E	20.5
V322	44V	33A			1.3
L468	27L	9A	4P	3V	3.0
E471	32E	31A			1.0
I472	38I	3A	4T	8V	12.7

participate in binding to coactivators. Similar relationships between roles in stability and binding were not seen in shotgun alanine scans of the human growth hormone−growth hormone receptor or the ErbB2−(anti-ErbB2) interfaces,[9,24] although an analogous relationship was found in the study of the Erbin PDZ domain and an interacting peptide.[25] In the PDZ study, several of the residues contributing to the surface involved in binding are significantly buried, similar to the case in PPARγ. We speculate that the number of intramolecular contacts made by residues that participate in binding determines the overlap between residues important to protein−protein interaction and those involved in folding.

Although F306, Q314, L318, K319, and I472 are necessary for PPARγ stability, they may still play an additional role in coactivator binding that is unrelated to their role in protein stability. L318, in particular, lies in the middle of the PPARγ−SRC1 interface, buries a large amount of surface area upon interaction with SRC1, and makes extensive van der Waals contacts with the coactivator peptide.[26] L318 is therefore likely needed both for the interaction with coactivators and for the stability of the receptor. F306, Q314, and I472 are on the periphery of the PPARγ−SRC1 interface. These three residues participate in van der Waals contacts with the coactivator, but they also make extensive interactions with other PPARγ residues. As a result, it is difficult to speculate on their contributions to coactivator binding versus their importance in PPARγ stability.

Our results indicate that the conserved charge clamp residues K301 and E471 are crucial for coactivator binding. Neither residue plays a significant role in PPARγ stability, yet both are highly conserved among clones surviving PPARγ−coactivator interaction selection. K301 appears to be the more important of the two amino acids and was completely intolerant to alanine or threonine substitutions (Tables 1 and 2). Although E471 is clearly important to coactivator binding, the different degree of conservation observed when selecting for interaction with TRAP220 versus SRC1 (wt/Ala ratios of >50 versus 15.8, respectively) suggests that its role in binding energetics varies depending on the context of the particular coactivator. The hypothesis that E471 may represent a coactivator specificity determinant is consistent with the findings of Burris and co-workers, who report that E471 is necessary for binding to the coactivator TIF2 but is entirely expendable for binding to the PGC-1α coactivator.[27]

The PPARγ residue whose contribution to coactivator binding changes the most between the two coactivators tested is L468. This residue is intolerant even to conservative substitution with valine when interacting with $TRAP220^{643-651}$ (wt/Ala ratio >50), yet mutation at this position is partially tolerated in the interaction with $SRC1^{686-700}$ (wt/Ala ratio = 6.8). The PPARγ/SRC1 structure indicates that L468 of PPARγ makes extensive contact with I689 of SRC1. Although not part of the canonical coactivator LXXLL motif, I689 immediately precedes this motif, and position 689 generally contains hydrophobic residues within NRIDs. Indeed, when I689 was randomized along with other residues of the $SRC1^{686-700}$ NRID, only those sequences containing Trp, Leu, or Met at residue 689 were selected on the basis of interaction with PPARγ (Figure 3). Alanine scanning of the coactivator revealed I689 of SRC1 to be relatively important for interaction with PPARγ (Ala/wt ratio of 14.5). Collectively, these results suggest that, in addition to the three leucines of the coactivator LXXLL motif, the residue just before this motif, together with PPARγ residue L468, are important for binding. Since both of these positions vary considerably between different NRIDs and NRs, the interaction between these residues may contribute significantly to the specificity between NRs and coactivators, consistent with a proposal of Yamamoto and co-workers.[28]

The three leucines of the LXXLL motif were the most highly conserved residues in the alanine scan of the $SRC1^{686-700}$ coactivator (Table 1). L690 and L694, the two most important coactivator residues for formation of the interface with PPARγ, interact directly with the PPARγ residues L318, K319, and I472.[26] This structural relationship is consistent with our observations that these three PPARγ residues are important for both coactivator binding and nuclear receptor stability. Our findings also suggest that H691 and R692 of SRC1, the two central residues of the L**XX**LL motif, play no significant role in binding PPARγ. Neither position exhibits any conservation in the SRC1 alanine scan, and both are actually disfavored relative to alanine substitution (wt/Ala ratios = 0.25 for H691 and 0.57 for R692). The high helical propensity of alanine may explain its preference at these positions and suggests that it may be possible to increase the affinity of designed coactivator sequences simply by choosing alanine at these positions instead of the corresponding natural residues.

Although the importance of the leucines within the LXXLL motif is not surprising, their exceptionally high level of conservation when randomized to any amino acid was unexpected (Figure 3). That LXXLL-containing sequences were enriched almost exclusively relative to isoleucine- or valine-containing variants such as LXXLV or IXXLI suggests that there is a large decrease in binding energy upon even conservative mutations to the canonical LXXLL motif.

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Although it is known that various coactivators display some functional specificity for particular nuclear receptors, how this specificity is determined is unclear. Given the high conservation of both the AF2 binding cleft of NRs and the LXXLL motif of coactivators, it has been commonly suggested that specificity of NR−coactivator interactions arises from factors other than the nature of the AF2−LXXLL interface, such as coactivator expression levels.[29] However, both coactivator residues that lie outside the LXXLL core yet were included in our alanine scan play significant roles in receptor binding. H687, which lies three amino acids before the LXXLL motif of SRC1, makes a significant contribution to the interaction with PPARγ (wt/Ala = 8.5), as does I689 (wt/Ala = 14.5), which was discussed earlier. These findings are consistent with a model in which residues outside the LXXLL motif are less highly conserved between coactivators and interact with less highly conserved regions of NRs yet still play important roles in NR−coactivator binding, resulting in their ability to control the specificity of NR−coactivator interactions. The importance of residues flanking the LXXLL motif in providing specificity between coactivators and NRs is consistent with the results of Darimont et al. and McInerney et al.[28,30]

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Conclusion

Using shotgun alanine scanning coupled with a modified fragment complementation selection for protein−protein interactions, a large binding epitope was identified in the nuclear receptor PPARγ, stretching between the charge clamp residues of K301 and E471 that mediates and the interaction between PPARγ and two peptidic coactivators. Slightly different epitopes were identified for the different coactivator sequences, suggesting that different coactivators can bind the AF2 cleft of PPARγ in nonidentical ways. A second assay examining the in vivo stability of the PPARγ variants generated by shotgun scanning demonstrated that many of the residues of the AF2 cleft are crucially important for the stability of the PPARγ and are largely intolerant to mutation. The immutability of this interface may be an unusual feature of PPARγ, or nuclear receptors in general, as similar intolerance to interface mutations was not found in related studies of protein−protein interactions.[9,24]

Although our scans focused on interactions between the core LXXLL motif and AF2 of PPARγ, several residues on the periphery of the receptor−coactivator interface, including PPARγ L468 and E 471 as well as SRC1 H687 and I689, appear to make significant contributions to binding in a coactivator-dependent manner. These and other interactions flanking the LXXLL motif may prove sufficient to determine NR/coactivator specificity either among natural coactivators or among future designed coactivator variants.

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