Quantitative Proteomics of the Thyroid Hormone Receptor-Coregulator Interactions* [S]

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The thyroid hormone receptor regulates a diverse set of genes that control processes from embryonic development to adult homeostasis. Upon binding of thyroid hormone, the thyroid receptor releases corepressor proteins and undergoes a conformational change that allows for the interaction of coactivating proteins necessary for gene transcription. This interaction is mediated by a conserved motif, termed the NR box, found in many coregulators. Recent work has demonstrated that differentially assembled coregulator complexes can elicit specific biological responses. However, the mechanism for the selective assembly of these coregulator complexes has yet to be elucidated. To further understand the principles underlying thyroid receptor-coregulator selectivity, we designed a high-throughput in vitro binding assay to measure the equilibrium affinity of thyroid receptor to a library of potential coregulators in the presence of different ligands including the endogenous thyroid hormone T3, synthetic thyroid receptor β-selective agonist GC-1, and antagonist NH-3. Using this homogenous method several coregulator NR boxes capable of associating with thyroid receptor at physiologically relevant concentrations were identified including ones found in traditional coactivating proteins such as SRC1, SRC2, TRAP220, TRBP, p300, and ARA70; and those in coregulators known to repress gene activation including RIP140 and DAX-1. In addition, it was discovered that the thyroid receptor-coregulator binding patterns vary with ligand and that this differential binding can be used to predict biological responses. Finally, it is demonstrated that this is a general method that can be applied to other nuclear receptors and can be used to establish rules for nuclear receptor-coregulator selectivity.

Thyroid hormone (3,5,3′-triiodo-l-thyronine, T3)1 regulates multiple physiologic processes in development, growth, and metabolism (1, 2). Most T3 actions are mediated through the thyroid hormone receptors (TR), which regulate transcription of target genes either positively or negatively in response to hormone binding. There are two different genes that express different TR subtypes, TRα and TRβ. Each transcript can be alternatively spliced generating different isoforms (TRα1, TRα2, TRβ1, TRβ2) (3, 4). While most of these isoforms are widely expressed, there are distinct patterns of expression that vary with tissue and developmental stage. In particular, TRβ2 is found almost exclusively in the hypothalamus, anterior pituitary, and developing ear. In addition, mice deficient in either TRα or TRβ display unique phenotypes, suggesting that the different TR isoforms have unique regulatory roles (5–10).

The thyroid hormone receptors belong to a superfamily of proteins known as the nuclear hormone receptors (NR). Like other members of the NR superfamily, TR has three functional domains: an N-terminal transactivation domain (NT), a central DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD) (11). The DBD of TR recognizes two types of DNA sequences: TREs and TRPs. TR can interact with a TRE as a monomer, homodimer, or heterodimer with the retinoid X receptor (RXR) (12). However, receptor activation from the heterodimer complex is the best characterized to date. Both liganded and unliganded TR bind to TREs. In the absence of T3, TR associates with corepressor proteins at the TRE maintaining the chromatin in a compact state and therefore repressing gene activation. Upon binding of T3, TR undergoes a conformational change releasing corepressor proteins and allowing for the interaction with coactivator proteins that enhance TRE-driven gene transcription.

Structural, biochemical, and genetic studies have provided a considerable amount of information about NR-coregulator interactions. The best studied coregulators belong to the p160 protein family of steroid receptor coactivators (SRC) (13). Members of this family include SRC1, SRC2 (GRIP1/TF2), and SRC3 (AIB1/TRAM1/RAC3/ACTR). These proteins contain several functional domains including the nuclear receptor interaction domain (NID), and two activation domains that interact with other coactivatory proteins CBP/p300 (AD-1) and CARM-1 (AD-2) (Fig. 1A). Within the NID there are three repeated motifs with the consensus sequence LXXLL, often termed the NR box. In addition there is a unique fourth LXXLL motif found in the extreme C terminus of an alternatively spliced variant of SRC1, SRC1-a. Several investigations have shown that the LXXLL motif is necessary and sufficient for interaction with NR (14, 15). Further structural work with a...
coregulator NR box peptide and liganded TR has revealed that the LXXLL binds to a hydrophobic groove in the TR-LBD as an α-helix (16). Other coregulators include CREB-binding protein (CBP)/p300 (17, 18), thyroid receptor-activating protein (TRAP)/vitamin D receptor-interacting protein (DRIPs)/peroxisome proliferating-activating receptor-binding protein (PPBP) (19, 20), androgen receptor activator 70/55 (ARR70/55) (21, 22), receptor-interacting protein 140 (RIP140) (23), PPARG coactivator 1 (PGC-1) (24), thyroid receptor-binding protein (TRBP)/PPAR-interacting protein (PRIP) (25, 26), DAX-1 (27), and small heterodimer partner (SHP) (28). The interaction of these coregulators with nuclear receptors is also mediated by LXXLL motifs. An analogous motif, L/XXH, has been identified in corepressors such as nuclear receptor corepressor (NCOR) and silencing mediator of retinoid acid (SMRT), and structural studies have shown that the binding sites for coactivators and corepressors partially overlap (29).

There is a large pool of coregulators available for interaction with TR. Although there appears to be some functional redundancy within the SRCs, there is also evidence that SRCs have distinct biological regulatory roles. While mice deficient in SRC1 exhibit resistance to thyroid hormone (RTH), the phenotypes for mice deficient in SRC2 and SRC3 are distinct with no evidence of RTH (30–32). These studies, along with recent work with the progesterone and glucocorticoid receptors, have demonstrated that interaction with specific coregulators can elicit specific biological responses (33). However, it remains unclear how NRs discriminate between different coregulators. In this study we sought to define rules that govern TR-coregulator selectivity.

Combinatorial peptide libraries have been used to define NR-coregulator specificity, and have revealed that the sequences immediately flanking the NR box are critical for specificity (34, 35). However the peptides in these studies were generated from random libraries that do not represent the true NR box sequences. Other investigations focused on defining SRC NR box selectivity using a subset of coregulator NR boxes from the SRCs. This work has shown that ligands can allosterically modulate the coregulator binding pocket and therefore differentially alter specific SRC recruitment and NR box usage (36–38). However, to date there has been no comprehensive study of the interactions of TR and natural coregulator NR boxes. To address this issue, we designed an in vitro binding assay to measure the equilibrium binding of TRβ to a library of potential coregulators in a high-throughput manner using fluorescence polarization. With this method, binding constants for TRβ to coregulator NR boxes were determined in a consistent format, including NR boxes from SRCS and nine other known coregulators. In addition the TRβ-coregulator binding patterns for three different ligands including T3, the synthetic TRβ-selective agonist GC-1, and the T3 antagonist NH-3 were defined. This quantitative information can be used to establish rules for TRβ coregulator selectivity, and these rules can be used for predicting biological responses.

EXPERIMENTAL PROCEDURES

Ligand Synthesis—GC-1 and NH-3 were synthesized as previously described (39, 40). Protein Expression and Purification—Human TRβ LBD (His6) residues Glu202–Asp232 was expressed from a PET28a construct (Novagen) in BL21 (DE3) (20 °C, 0.5 mM isopropyl-1-thio-β-D-galactopyranoside added at OD600 = 0.6) as previously described (15). Cells were harvested, resuspended in sonication buffer (20 mM Tris, 300 mM NaCl, 0.025% tween, protease inhibitors, 10 mg of lysozyme, pH 7.5, 30 min on ice), and sonicated for 3–5 min on ice. The lysed cells were centrifuged at 100,000 × g for 1 h, and the supernatant was loaded onto Talon resin (Clontech). Ligated protein was eluted with 500 mM imidazole plus ligand (3′,5′-triiodo-L-thyronine (Sigma); GC-1; or NH-3). Protein purity was assessed by SDS-PAGE and HPSEC, and protein concentration measured by the Coomassie protein assay.

Peptide Library Synthesis—Coregulator peptides consisting of 20 amino acids with the general motif of CXXXXXXXLXXLAI/AXXX-X-XXX were constructed, where C is cysteine, L is leucine, A is alanine, and X is any amino acid. Of the all coregulator peptides were obtained from human isoform candidate genes (SRC1/AA53053, SRC1/AA535596, SRC3/AA519092, TRAP220/Q81021, TRAP100/Q16548, TRBP/Q146868, TRAP100/Q75448, ARA70/Q13772, ARA55/ NP_057011, p300/Q92831, RIP140/P48532, DAX-1/I518453, SHP/Q15466). The peptides were synthesized in parallel using standard fluorenylmethoxycarbonyl (Fmoc) chemistry in 48 well synthesis blocks (FlexChem System, Robins) (41). Preloaded Wang (Novagen) resin was washed with 20% acetonitrile in dimethysulfoxide (DMSO). The next amino acid was then coupled using 2-(4H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (2.38 eq), Fmoc-protected amino acid (2.5 eq), and diisopropylethylamine (5 eq) in anhydrous dimethylformamide. Coupling efficiency was monitored by the Kaiser test. Synthesis then proceeded through a cycle of deprotection and coupling steps until the peptides were completely synthesized. The completed peptides were cleaved from the resin with concomitant side chain protection (81% trifluoroacetic acid, 5% phenol, 5% thioisobutyric acid, 2.5% ethanedithiol, 3% water, 2% dimethylsulfoxide, 1.5% ammonium iodide), and the crude product was evaporated using a Speedvac (GeneVac). Reversed-phase chromatography followed by mass spectrometry (MALDI-TOF/ESI) were used to purify the peptides. The purified peptides were lyophilized. A thiol reactive fluorophore, 5-isothiocyanatofluorescein (Molecular Probes), was then coupled to the N-terminal cysteine following the manufacturer’s protocol. Labeled peptide was isolated using reversed-phase chromatography and mass spectrometry. Peptides were quantified using UV spectroscopy. Purity was assessed using LCMS (Supplemental Material).

Direct Binding Assay—Using a BiomekFX in the Center for Advanced Technology (CAT), mTRβ-LBD was serially diluted from 70–0.002 μM in binding buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol) containing 140 μM ligand (T3, GC-1, or NH-3) in 96-well plates. Then 10 μl of diluted protein was added to 10 μl of fluorescent coregulator peptide (20 μM) in 384-well plates yielding final peptide concentrations of 35–0.001 μM and 10 nM fluorescent peptide concentration. The samples were allowed to equilibrate for 30 min. Binding was then measured using fluorescence polarization (excitation λ 485 nm, emission λ 530 nm) on an Analyst AD (Molecular Devices). Two independent experiments were assayed for each state in quadruplicate. Data were analyzed using SigmaPlot 8.0 (SPSS, Chicago, IL), and the Kajan values were obtained by fitting data to the following equation (y = min + (max − min)/1 + (x/Kajan) Hill slope).

RESULTS

Coregulator Peptide Library—A library of known coregulator peptides consisting of the LXXL sequence plus 7–8 additional flanking residues at each terminus was synthesized (Fig. 1B). Previous screens with coactivator peptides established amino acid residues at +6 to +12 as critical for binding (38, 42). To capture these specificity determinants, peptides of 20-amino acid length were generated. Additionally, negative control peptides were made for each coregulator NR box by replacing L+4 and L+5 with alanine (LXXAA), as this substitution has been shown to abolish interactions with NR (38). A thiol reactive fluorescent probe was covalently attached to each peptide via a cysteine positioned at the N terminus of each peptide. The coregulator peptides are listed in the far left column of Fig. 1B, where SRC1-1, SRC1-2, SRC1-3 represent the first, second, and third NR boxes in SRC1, respectively. This nomenclature, first proposed by O’Malley, is applied to all of the coregulator peptides studied throughout this report (30). All peptide probes were synthesized in parallel using the Fmoc strategy and purified by RP-HPLC. Identity and purity were confirmed using HPLC and MALDI-TOF or LCMS (Supplemental Information). Seven targeted coregulator NR boxes, TRAP100-1, TRAP100-5, RIP140-2, RIP140-4, TRAP220-1(−), TRBP-1(−), and TRAP100-6(−), are omitted as they could not be synthesized or purified after several attempts.
Coregulator Peptides Bind to hTR\(\beta\)-LBD in Four Different Binding Modes—Initial peptide binding studies were carried out with SRC2-2, and the ligand binding domain of the human thyroid hormone receptor \(\beta\) (hTR\(\beta\)-LBD) in the presence of T3. As shown in Fig. 2A, SRC2-2 binds to TR\(\beta\) in a saturable dose-dependent manner with a measured \(K_d\) of 0.7 \(\mu\)M, consistent with literature reports (0.8 \(\mu\)M) (15). We also confirmed that this interaction was specific by carrying out binding studies with a mutated SRC2-2 peptide (LXAA, SRC2-2(\(\Delta\)XX)). The trace observed in Fig. 2A reveals that SRC2-2(\(\Delta\)XX) does not interact with TR\(\beta\) in the presence of T3.

To further elucidate the coregulator binding pattern of TR\(\beta\) in the presence of T3, direct binding assays were carried out with the entire coregulator peptide library. This set of experiments was executed by maintaining a constant concentration of coregulator peptide (10 nM) and varying the TR\(\beta\) concentration from 0.001–35 \(\mu\)M in the presence of saturating amounts of T3 (90 \(\mu\)M). The results revealed that the 34 different coregulator peptides bound to liganded TR with varying degrees of affinity. Individual Klotz plots were constructed for each coregulator peptide. This analysis revealed four different binding modes. Example equilibrium affinity curves are summarized in Fig. 2(A–D) and \(K_d\) value ranges are reported in Fig. 3A. In no case did the negative peptide controls bind to liganded TR\(\beta\).

The first binding mode consisted of peptides that bound in a dose dependent and saturable manner (Fig. 2A) where a clear plateau was reached within the protein concentration range studied. Eight coregulator peptides exhibited this mode: SRC1-1, SRC1-2, SRC1-3, TRAP220-1, TRBP-1, p300, RIP140-5, and DAX1-3. The \(K_d\) values for this class ranged from 0.7 to 10 \(\mu\)M.

The next binding mode included peptides where binding...
that this is a saturable binding curve where a measurable $K_d = 0.7 \, \mu M$ is extracted by fitting to the equation $(y = \min + (\max - \min)/1 + 1/x K_d)$ Hill slope. The binding of TRβ to the negative control SRC2-2 peptide is also shown in red. Panel B, the binding curve for SRC1-3 is shown in light green. This binding curve appears to be reaching saturation, but no visible plateau is observed. The $K_d$ is reported as 10.1–30 $\mu M$. Panel C. The binding curve for SRC3-3 is shown in grey. Polarization is increasing with TRβ concentration but does not appear to be reaching saturation. The $K_d$ is reported as >30 $\mu M$. Panel D, the binding curve for TRAP220-2 is plotted in red indicating that no binding is observed. The solid circles denote the native NR box peptides, solid lines represent fitted curves for native NR box peptides, open red circles represent negative NR box peptides (LXXAA), and the dashed red line is the fitted curve for the negative NR box peptide.

Fig. 2. TRβ-coregulator binding isotherms in the presence of T3. Panel A, the binding of TRβ SRC2-2 is plotted in dark green to signify that this is a saturable binding curve where a measurable $K_d = 0.7 \, \mu M$ is extracted by fitting to the equation $(y = \min + (\max - \min)/1 + 1/x K_d)$ Hill slope. The binding of TRβ to the negative control SRC2-2 peptide is also shown in red. Panel B, the binding curve for SRC1-3 is shown in light green. This binding curve appears to be reaching saturation, but no visible plateau is observed. The $K_d$ is reported as 10.1–30 $\mu M$. Panel C. The binding curve for SRC3-3 is shown in grey. Polarization is increasing with TRβ concentration but does not appear to be reaching saturation. The $K_d$ is reported as >30 $\mu M$. Panel D, the binding curve for TRAP220-2 is plotted in red indicating that no binding is observed. The solid circles denote the native NR box peptides, solid lines represent fitted curves for native NR box peptides, open red circles represent negative NR box peptides (LXXAA), and the dashed red line is the fitted curve for the negative NR box peptide.

The third binding mode is one in which polarization increases with protein concentration but does not seem to be reaching saturation. This mode is exemplified by SRC3-3 (Fig. 2C) where the polarization of SRC3-3 slowly increases with TRβ concentration. Other coregulators included in this category are SRC1-1, SRC3-1, SRC3-2, TRAP220-2, TRBP-2, and ARA70. To accurately obtain $K_d$ values, however, the binding studies would need to be carried out at protein concentrations varying from 1–300 $\mu M$ as this would give the widest range of polarization values. Working with TRβ protein concentrations higher than 100 $\mu M$ is problematic due to protein aggregation and decreased protein stability. In order to reflect the inability to obtain an unambiguous $K_d$ value, we report a $K_d$ range, 10–30 $\mu M$, for coregulator peptides that exhibit this binding mode.

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teins such as AP-1, CRE, and NFκB-response element (25). There are two LXXLL motifs in TRBP and both can interact with TRβ. Our studies indicate that TRBP-1 is preferentially recruited to TRβ in the presence of T3.

RIP140 is a coregulator that contains 9 LXXLL motifs and has been shown to interact with many NRs including TRβ (23). It has been suggested that RIP140 directly competes with other coregulators (23). Unlike traditional coregulators, however, RIP140 represses transcription upon binding to NR (46–49). Here we show liganded TRβ has a clear preference for three of the NR boxes in RIP140, RIP140-3, RIP140-5, and RIP140-8. One NR box peptide in particular, RIP140-5, bound fairly tightly with a $K_d$ of 2.5 μM ± 0.4.

Three additional coactivators, p300, ARA70, and DAX1, were also shown in this report to associate with TRβ with varying degrees. ARA70 and DAX1 had not previously been investigated for their interaction with TRβ.

**Ligands Alter Coregulator Recruitment**—To investigate ligand effects on coregulator recruitment, binding studies were performed in the presence of the TRβ selective agonist, GC-1. GC-1 is a halogen-free thyromimetic that is ~10-fold selective for binding to TRβ versus TRα (39). It has been shown that the oxyacetic acid at the carbon-1 position (Fig. 3A) is responsible for the selective TRβ binding of GC-1 (50). Additionally, crystallographic studies have confirmed that the oxyacetic acid group participates in a hydrogen bonding network in the TRβ LBD polar pocket (51). We sought to determine how these interactions might alter coregulator specificity.

In the presence of GC-1 the coregulator peptides bound to TRβ with varying degrees of affinity and all four binding modes were observed. Overall the coregulator binding patterns for GC-1 and T3 were similar in terms of which coregulator peptides were recruited. However, the degree to which they bound varied. In most cases, the coregulator peptides bound with similar or slightly lower affinity to TRβ-GC-1 than to TRβ-T3. Several NR boxes, particularly those of the SRC family, exhibited significant differences in affinity to TRβ-GC-1 relative to TRβ-T3. All of the SRC2 NR boxes bound TRβ-T3 in a measurable $K_d$ range, whereas in the presence of GC-1 a saturated binding curve was only observed for SRC2-2. Additionally, SRC1-2 appears to be much more strongly recruited by TRβ-T3, whereas the opposite is true for SRC3-3. Other notable differences between T3 and GC-1 were seen with TRAP220 and TRBP where recruitment decreased in the presence of GC-1.

In addition to studying agonist induced coregulator recruitment, we wanted to explore how antagonists may affect coregulator binding. The recently reported T3 antagonist NH-3 (Fig. 3A) was tested against the entire coregulator peptide library.
The NRs show a clear preference for particular NR boxes. One factor that drives this specificity is the amino acid residues immediately flanking the NR box (15, 34, 36–38). Most prior work focused on individual coregulators or the p160/SRC coregulator family (15, 19, 23, 25, 38, 43–45, 52). To expand our understanding of coregulator recruitment, we investigated the ability of TRβ to bind to a library of known coregulator NR boxes using a homogenous equilibrium binding assay. The results from this screen demonstrate that the coregulator binding pattern for TRβ is distinct from other NR (ERα, ERβ, AR, data not shown) and new TRβ-coregulator peptide interactions, including RIP140-5 (Kd = 2.5 μM), ARA70 (Kd = 10–30 μM), and DAX1-3 (Kd = 3.6 μM) were identified. The NR box peptides that interacted with TRβ revealed specificity elements including a propensity for hydrophobic groups at the −1 position and for proline at the −2 position as seen in TRAP220 and TRBP-1 (20, 45). Additional observations may be partially explained by the selective binding of RIP140 and DAX1. In our studies we find that both TRβ-T3 and TRβ-GC-1 strongly interact with RIP140-5 and DAX1-3, but TRβ-NH-3 fails to recruit these coregulators. From these observations, it can be predicted that T3 and GC-1 can repress gene transcription but NH-3 lacks this ability. Thus NH-3 treatment may result in partial activation of genes that are normally repressed by TRβ-T3. If this is the case, then NH-3 would display unique pharmacology by blocking ligand activation of post-positively regulated T3-responsive genes and causing derepression of negatively regulated T3-responsive genes.

Presumably there are additional factors that influence NR recruitment of coregulators such as post-translational modifications, structural determinants arising from specific DNA response elements, cooperativity, cellular environment, and additional interaction surfaces on the NR and coregulator proteins.

To fully dissect NR-coregulator interactions, more complex models will need to be developed. The use of full-length molecules for determining NR-coregulator binding affinities has been employed with the estrogen receptors and members of the SRC family (60). Although this work demonstrated that the binding affinities are 3–5 fold higher than predicted with coregulator peptides and NR-LBD, the overall selectivity of ER isoforms for SRC members was consistent with previous investigations. This emphasizes the utility of a simple affinity model as a first step for establishing rules of NR-coregulator selectivity.

NR signaling is a multivariable complex process that utilizes differences in NR, NR isoforms, a diverse set of coregulators, ligands, tissue variability, and unique DNA response elements. It remains unclear how this protein network can potentiate signals for specific biological responses. However one point of regulation may derive from specific NR-coregulator interactions. Using an equilibrium binding assay, the binding affinities of TRβ for a large set of NR boxes in the presence of multiple ligands were quantitatively determined and some rules were defined that account for the specificity of these interactions. Additionally, it was shown that these binding patterns could be used to predict biological responses. Finally, we believe that this method may be generalized to other nuclear receptors to establish patterns of NR-coregulator selectivity.

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