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Structural mechanisms in the regulation of the thyroid hormone reeptor

Author
Wagner, Richard Lawrence

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Structural Mechanisms in the Regulation of the Thyroid Hormone Receptor

Richard Lawrence Wagner III

Biophysics

For my Father

The text of Chapter One was originally published as "A structural role for hormone in the thyroid hormone receptor", in the December 15th, 1995 issue of *Nature* (**378:** 670-697).

The text of Chapter Two was published in similar form as "Structure and specificity of nuclear receptor: coactivator interactions", in the November 1998 issue of *Genes and Development* (**12**(21): 3343-3356).

Appendix One consists of secondary papers on topics directly related to those described in the thesis, collected here to enhance its value as reference work. These include:

"Preliminary crystallographic studies of the ligand-binding domain of the thyroid hormone receptor complexed with triiodothyronine," by M.E. McGrath *et. al.*, originally published in the March 25, 1994 issue of the *Journal of Molecular Biology* (**237**(2): 236-239.)

"A natural transactivation mutation in the thyroid hormone beta receptor: impaired interaction with putative transcriptional mediators," by T.N. Collingwood, *et al.*, originally published in the January 7, 1997 issue of the *Proceedings of the National Academy of Sciences U S A* (**94**(1): 248-53).

"Hormone-Dependent Coactivator Binding to a Hydrophobic Cleft on Nuclear Receptors," by W. Feng, *et. al.*, originally published in the June 12, 1998 issue of *Science* (**280**: 1747-1750.)

Permissions to reproduce previously published works are collected in Appendix Three.

The text of Chapter Two describes a collaboration between Beatrice Darimont and Richard Wagner. The biochemical experiments described are the work of Beatrice Darimont, and the structural results are the work of Richard Wagner. Both individuals were involved in the discussion and interpretation of the experiments, and collaborated in writing the manuscript. As an equal contributor to the work, the material is suitable for inclusion in his dissertation.

Robert J. Fletterick, Ph.D

Chair, Thesis Committee

ABSTRACT

Structural Mechanisms in the Regulation of the Thyroid Hormone Receptor

by Richard Lawrence Wagner III

Nuclear receptors, members of a superfamily of eukaryotic transcription factors, regulate gene expression in response to small, hydrophobic ligands. The receptors exhibit a modular structure, with functionally separable domains. One of the most highly-conserved domains is the ligand-binding domain (LBD). The ligand binding domain adopts distinct conformations as apo-receptors, agonist-bound or antagonist-bound species, which support or preclude interactions with proteins such as transcriptional corepressors and transcriptional coactivators.

To understand how hormone exerts its control over the activity of the nuclear receptors, we determined the crystal structure of the liganded thyroid hormone receptor LBD, alone and in complex with a peptide from GRIP-1, a p160 coactivator. In these structures, the ligand is completely buried within the domain as part of the hydrophobic core. The conserved leucine residues of the p160 coactivator bind a highly conserved hydrophobic cleft formed by helices H3, H4, H5, and H12 of the TR LBD, defining the activation function AF-2 within the LBD. Similarity between the NR-box peptide in the cocrystal, and α-helix H12 in the structure of the raloxifene-bound estrogen receptor (ER) LBD, suggests the antagonist-bound LBD fails to interact with p160 coactivators by eliminating the part of the coactivator interaction surface that involves H12, and occluding the remaining part of the coactivator interaction surface. These observations suggest a structural role for hormone, in establishing the active conformation of the receptor, that is likely to underlie hormonal regulation of gene expression for the nuclear receptors.

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Introduction

All men naturally have an impulse to get knowledge. A sign of this is the way we prize our senses; for even apart from their utility, they are prized on their own account, especially sensing with the eyes. For not only from their practical motives, but also when we have nothing practical in view, we could be said to prefer sight to any of the other senses. The reason is that of all the senses it can best bring us knowledge, and best discerns the many differences among things.

Aristotle, Metaphysics

In the opening of the *Metaphysics* , Aristotle puts forth a philosophy of man as a rational animal, which he supports using sight as an example of our natural curiousity. That same impulse - to know - must explain the fascination of the biologist with experimental techniques such as X-ray crystallography. Why? because X-ray crystallography is visual, revealing its object through the phenomenon of diffraction. The image thus obtained offers insight into function and mechanism (Buchbinder *et al.*, 1998; Sprang 1997); more important, it dispels false ideas and engenders new questions (Bourguet *et al.*, 1995). And when the truth revealed is so unexpected, so elegant, to allow us a brief glimpse of the mysterious (Xu *et al.*, 1997; Sicheri *et al.*, 1997) - then one enjoys the highest aspiration of knowledge.

Signalling

The genome of the cell is the repository of its possibilites, from which it chooses by selectively expressing its genes. *Signalling* describes the biological process by which external cues are transduced into an altered state of the cell, primarily through an alteration in gene expression.

Prokaryotes provide several examples of signalling in response to environmental cues, such as nutritional deficiencies (Yanofsky and Crawford, 1987; Beckwith, 1987). The study of these primitive systems revealed the basic requirements for regulation of gene expression: a regulatory element, or response element, within the target gene; and a transcriptional regulator (Jacob and Monod, 1961). The response element, a specific DNA sequence within the regulatory region of the gene recognized by the transcriptional regulator, confers responsiveness to the signal on the gene. The transcriptional regulator, a protein, binds the response element, turning the gene on or off in response to the signal. In the simplest systems, the transcriptional regulator also binds the signal molecule, so that the signal and the change in transcription are directly linked.

In eukaryotic signalling, that basic mechanism becomes elaborated as a signalling network, in which multiple proteins act in discrete steps to transduce the external signal into altered gene expression. The sequence of events coupling the initial signal to its final genomic effect can be very complex, providing opportunites for amplification of the signal, for modulation of the biological response, and for interaction with other signalling networks. The protein components involved in signal transduction networks share two obligatory functional parts: a receptor, to receive the signal; and an effector, to mediate the response. Additionally, two other functional parts may be present: a modulator, to tune the response

and allow coordination with other signalling networks; and finally a timer, to adjust the duration of the signal (Bourne, 1988). The structural mechanisms that couple these functional parts, permitting the protein to transduce the signal, are described by allostery.

Allostery

Allostery, or regulation of function through structural change, was proposed by Monod and Jacob to explain how binding of one regulatory molecule could influence the affinity of a protein for a second molecule (Tomkins and Yielding, 1961; Monod *et al.*, 1963). Because allostery does not depend on a particular chemistry, merely upon structural complementary between the protein and its regulatory molecules, any regulatory pathway could be constructed using allostery. In this sense, all signal transduction pathways are similar, as they share this fundamental mechanistic principle.

The elucidation of an allosteric regulatory mechanism requires a description of the protein in complex with its regulatory ligands. Therefore, a fundamental understanding of the signal transduction process requires the application of structural techniques to obtain an atomic level description of the signalling events. Examples of structural changes in signal transduction proteins illustrate the two classes of allosteric transitions : changes in subunit orientation/oligomeric state; and local refolding.

subunit orientation/oligomeric state

Growth factors, such as growth hormone, and cytokines such as erthyropoietin, initiate signal transduction by binding to the extracellular domain of specific single-transmembrane-segment receptors. Hormone binding promotes receptor dimerization, leading to phosphorylation of the intracellular kinase catalytic domain and activating a

kinase cascade. The asymmetric hormone binds identical sites in the receptor dimer, using distinct binding faces; driving the association are favorable hydrophobic contacts between the hormone and receptor. Differences in receptor subunit orientation between the complex of erthyropoietin receptor with erthyropoietin, and erthyropoietin receptor with a symmetric peptide mimetic, argues that efficient signalling requires a particular stereochemical orientation in addition to oligomerization, and by extension that a hormone antagonist could block response by trapping the dimer in an unproductive subunit orientation (Syed *et al.*, 1998; Livnah *et al.*, 1996).

local refolding

G proteins are a superfamily of GTP hydrolases that participate in many signal transduction pathways, such as those initiated by seven-transmembrane-segment receptors and receptor-tyrosine kinases. In all G-proteins, the binding and hydrolysis of GTP triggers conformational changes in specific regions of a catalytic domain; these plastic regions are termed switch I, switch II, and in heterotrimeric G-proteins, switch III. In the GTP-bound state, the G-protein is active, and interacts with effector proteins through a binding site composed of switch II, and variously switch I and switch III. Presence of the nucleotide stabilizes the active conformation of the switch regions, through participation in the coordination of the Mg^{2+} ion (switch I), hydrogen bonding to the γ-phosphate of GTP (switch II), and ionic interactions between complementary residues in switches II and III. GTP hydrolysis severs these interactions, inducing a helix-coil transition at the N-terminus of switch II and reorienting the adjacent α-helix. Concomitant movements in switches I and III together dismantle the effector-binding site, inactivating the G protein (Sprang 1997).

Nuclear Receptors

In multi-cellular organisms, cells differentiate to assume specific roles. But cellular specialization poses a challenge in coordinating the activities of individual cells, if the organism is to enjoy the advantage from this division of labor. Hormones are signalling molecules that communciate between cells. Tomkins rationalized hormones as physiological symbols, signifying a particular metabolic state to the cell (Tomkins, 1975). But in contrast to the simple signalling pathways mentioned above, in which a specific metabolite directly interacted with an effector to regulate its own synthesis, hormones act through a specific receptor and regulate diverse and chemically unrelated processes. The receptor serves an adaptive function, conveying specificity in regulation (e.g. through selective DNA binding), while permitting a range of regulatory action, beyond that available to the limited chemistry of the hormone (Yamamoto *et al.*, 1999)

The intracellular hormones are small, lipophilic molecules including the steroids, retinoids, and thyroid hormone,which bind to receptors within the cell. These hormones exhibit diverse cellular actions. For example, the set of genes regulated by a particular steroid differs between cell-types; furthermore, within a particular cell-type, the steroid exerts both positive and negative regulation, turning on one set of genes and turning off another. Strikingly, in some cells possess an enabling function: the presence of the steroid is required for other signalling pathways to activate certain genes. The action of steroids, then, is specific, in that a particular subset of genes is affected; pleiotropic, in that multiple genes in a cell are affected; and combinatorial, in that the expression of particular target genes is coordinated with other signalling pathways.

The receptors for the intracellular hormones are self-contained signal transduction pathways - signalling in microcosm. The receptors receive the primary regulatory input, the

hormone; bind DNA; alter transcription; and interact with other signalling pathways. Consequently, the proteins possess multiple receptor, effector, and modulator functional parts within a single protein (Figure I-1). The coupling of these functional parts, to produce the regulatory properties described above, is the essence of intracellular receptor mechanism.

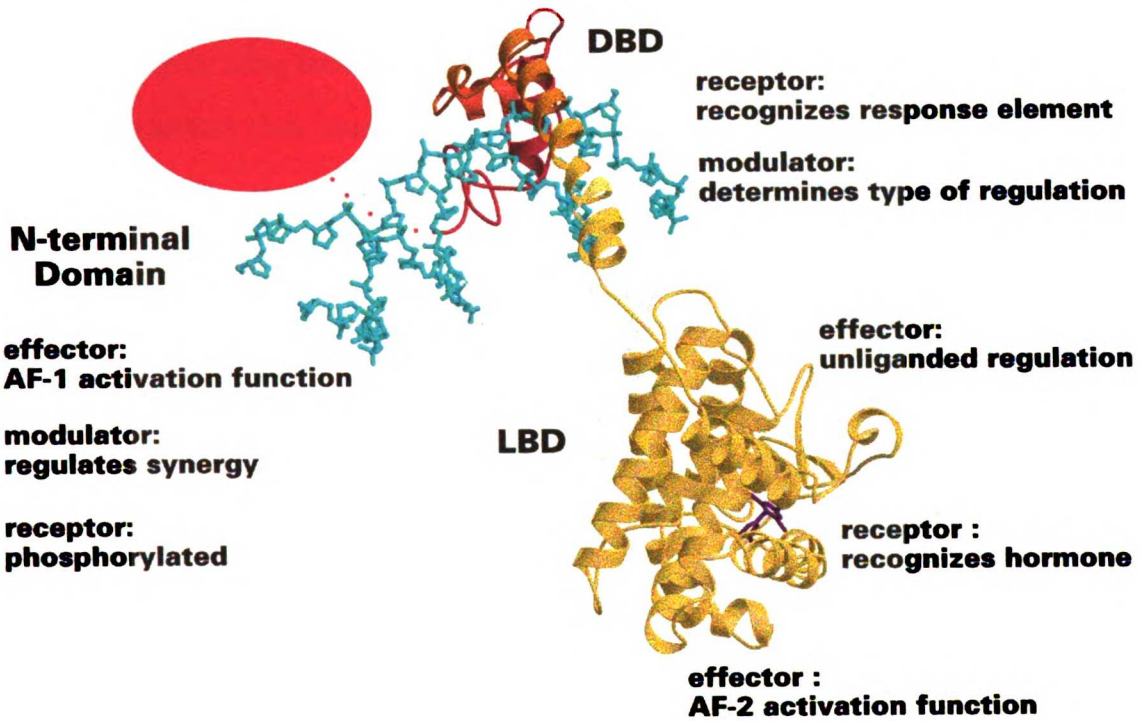
The most highly conserved domains within the intracellular receptor superfamily are the DNA-binding domain (DBD), and the ligand-binding domain (LBD)[1].. Both domains contribute to gene regulation. The DBD recognizes the response element; the presence of an element in the promoter of a gene confers potential hormone inducibility on that gene. Furthermore, the DBD can function as a modulator of the activity of the receptor, by adopting conformations that determine the type of regulation for the gene: functioning, in effect, as a "receptor" for an allosteric DNA "ligand" (Lefstin and Yamamoto, 1998). The LBD recognizes the cognate hormone, and regulates receptor function by adopting ligand-dependent conformations. These conformations alter the affinity of the receptor for various regulatory proteins that restrain the receptor (heat-shock proteins), that mediate its effects on the expression of the target gene (co-activators, co-repressors, or proteins that link to / function as part of the basal transcriptional machinery), or which participate in other signalling networks (Moras and Gronemeyer, 1998; Fondell *et al.*, 1996; Rachez *et al.*, 1998).

Figure I-1 Functional parts of an intracellular receptor

Domains of an intracellular receptor, classified according to functional role. The figure is intended to be representative, and does not reflect a particular receptor. References given in text.

[1] Other recognized domains include an N-terminal regulatory domain, a hinge domain between the DBD and LBD, and a C-terminal domain. Across the receptor superfamily, these domains show little sequence conservation; however, these domains do, influence gene regulation for a given nuclear receptor.

Figure I-1 Functional parts of an intracellular receptor



The work described here applies structural techniques to the allosteric interaction of receptor with its hormone, to understand how that interaction governs the conformation, and thus the activity, of the receptor. Chapter One, "A structural role for hormone in the thyroid hormone receptor," describes the structure of the rTRα LBD bound with an agonist, 3,5-dimethyl-3'-isopropylthyronine, and suggests how hormone might exert its control over the conformation of the receptor: hormone completes the hydrophobic core of the active receptor. The finding is notable in two respects. First, it was unexpected. Proteins abhor cavities, typically binding ligand at the interface between domains (hexokinase), using the loops connecting several α-helices or β-strands (α/β barrel), or within a surface cleft (class I MHC)(Branden and Tooze); but these receptors bound hormone within an interior cavity! Second, allosteric changes are usually described by rigid-body movements, as in the example of single-transmembrane-segement receptors, or local conformation changes coupled to altered hydrogen bonding, as in the example of G proteins. Hydrophobic residues, while recognized as important to the allosteric changes, are nevertheless ignored in many analyses (Sigler 1992). As a mechanism for allosteric regulation, rearrangements within the hydrophobic core of the protein may be relevant for a wide class for signal transduction proteins, and also provides a fresh perspective in reexamining classic allosteric proteins, even such a well-studied example as glycogen phosphorylase (Lin *et al.*, 1997).

However, even this fundamental allosteric interaction:

$$\text{receptor} + \text{hormone} = ?$$

does not define a unique activity. For example, hormones are classified in common parlance as agonists or antagonists, according to whether the ligand activates or blocks a response. But an agonist can produce opposite transcriptional effects in different contexts (e.g. the glucocorticoid receptor (GR) responds positively to an agonist, dexamethasone,

on a simple GRE, but negatively on the plfG composite element (Yamamoto *et al.*, 1992, and references therein)); as can an antagonist (e.g. the estrogen receptor (ER) does not respond to tamoxifen on an ERE-containing promoter, but increases transcription from an AP-1-containing promoter (Paech *et al.*, 1997)); and both agonists and antagonists can produce opposite transcriptional effects in a given promoter context in closely-related receptors (e.g. the estrogen receptor α-isoform (ERα) bound to estradiol increases transcription from an AP-1-containing promoter, while the β-isoform (ERβ) bound to estradiol reduces transcription from the same promoter (Paech *et al.*, 1997)). Other signals, such as phosphorylation, can activate certain receptors (e.g. phosphorylation of Tyr 537 of the ERα produces a constitutively active receptor (Arnold *et al* , 1997; Weis *et al.*, 1997)). Conversely, a liganded receptor can produce varied, even opposite, transcriptional effects in a given promoter context, subject to the particular composition of crucial regulatory proteins favored by the state of the cell (e.g. the GR either activates or represses transcription from the plfG composite element depending on the composition of the AP-1 multimer (Yamamoto *et al.*, 1992, and references therein)). These examples demonstrate that, superimposed upon a fundamental interaction, are an array of possible responses.

Recognizing, as illustrated above, that a particular conformation of the receptor does not itself determine the type of regulation, the work proceeds to examine the interaction of the hormone-bound receptor with a particular regulatory protein. Chapter Two, "Structure and specificity of nuclear receptor : coactivator interactions," directly addresses the interplay of receptor, hormone, and regulatory cofactor, by examining the interaction of the TR and GR with a p160 family coactivator, GRIP-1. The study describes the structure of the liganded thyroid hormone receptor LBD in complex with a peptide containing an NR box from the coactivator GRIP1, and includes an elegant biochemical analysis of the interaction between GRIP1 and the thyroid hormone receptor and glucocorticoid receptor done by Beatrice Darimont. By describing the receptor:coactivator interaction, the study shows how

different hormones can produce conformations that support or prevent the interaction with p160 coactivators. By suggesting sources of specificity in the interaction with p160 coactivators and various nuclear receptors, the study shows how related proteins can utilize the identical surface for interaction, but with different regulatory outcomes.

As it is physically unlikely that a given hormone can produce multiple conformations of the receptor, specificity in the p160 coactivator interaction allows for an interpretative role for the coactivator, extending the unique specification of activity one step beyond the hormone/receptor itself. (H. Ingraham, pers. comm.; D. Leitman, pers. comm.) Such an interpretative role, played by a factor external to the receptor, satsifies the most challenging characteristic of receptor signalling, its combinatorial nature. The association of the coactivator is governed by the physical constraints imposed by the interaction of the hormone with the receptor, as described in Chapter One, and consistent with the role of hormone as the primary input. But by defering the unique specification of activity, diverse transcriptional effects are possible through integration - depending the response element; the promoter context; even on the state, or identity, of the p160 coactivator -

Chapter One

A Structural Role for Hormone in the Thyroid Hormone Receptor

ABSTRACT

The crystal structure of the rat α_1 thyroid hormone receptor ligand-binding domain bound with a thyroid hormone agonist reveals that ligand is completely buried within the domain as part of the hydrophobic core. In addition, the C-terminal activation domain forms an amphipathic helix, with its hydrophobic face constituting part of the hormone binding cavity. These observations suggest a structural role for ligand, in establishing the active conformation of the receptor, that is likely to underlie hormonal regulation of gene expression for the nuclear receptors.

INTRODUCTION

The nuclear receptors, members of a superfamily of eukaryotic transcription factors, regulate gene expression in response to binding small, hydrophobic ligands (Evans 1988; Tsai and O'Malley 1994; Ribeiro *et al.* 1995). The receptors exhibit a modular structure, with functionally separable domains. The most highly conserved domains are the DNA-binding domain (DBD), and the ligand-binding domain (LBD) (Evans 1988; Laudet *et al.* 1992). The DBD recognizes the specific DNA response element for the receptor (Evans 1988; Glass 1994; Tsai and O'Malley 1994). Several structures of receptor DBDs have been determined, and many principles governing DNA recognition have been established (Luisi *et al.* 1991; Schwabe *et al.* 1993; Rastinejad *et al.* 1995). The LBD participates in several activities, including hormone-binding, homo- and/or heterodimerization, heat-shock protein complex formation, and transcriptional activation and repression (Tsai and O'Malley 1994; Ribeiro, Kushner et al. 1995). Importantly, hormone binding induces changes in receptor conformation that control these properties, and influence gene expression (Tsai and O'Malley 1994; Ribeiro, Kushner et al. 1995). Therefore, the three-dimensional structure of a liganded LBD is critical for understanding the structural mechanisms of hormone action.

Nuclear receptors for the thyroid hormones, of which 3,5,3'-triiodo-L-thyronine (T_3) is the most important in mammals, regulate the transcription of gene products that mediate hormone effects on differentiation, development, and metabolism (Ribeiro *et al.* 1995). Two distinct genes, α and β, produce thyroid hormone receptors (TRs) (Ribeiro, Apriletti et al. 1995). We report here the crystal structure of the LBD from the rat α_1 TR isoform (rTRα_1) with ligand bound at 2.2Å resolution. The structure shows that hormone completes the hydrophobic core of the active conformation of the receptor. In addition, the C-terminal ligand-dependent activation domain contributes to hormone binding, allowing

direct regulation by hormone. The recent publication of the crystal structure of the unliganded human α retinoid X receptor (hRXRα) (Bourguet *et al.* 1995), another nuclear receptor, provides an opportunity to consider differences between the two structures which may result from hormone binding . These differences suggest mechanisms through which hormone regulates TR function. We expect the structural role for ligand revealed in this study, and the control which that role provides over key functional elements of the receptor, to be general for the entire class of nuclear receptors.

RESULTS

Structure determination and refinement

A fragment of the rat α_1 TR isoform (residues Met122-Val410), beginning immediately after the DBD and containing the entire LBD, was expressed in *E. coli* using a T7 polymerase based expression system (Apriletti *et al.* 1995) (Figure 1.1). Selective purification of the bound rTRα_1 LBD was prepared through hydrophobic interaction chromatography of receptor saturated with ligand. Cocrystals of the rTRα_1 LBD, with either 3,5,3'-triiodothyronine (T_3) , 3'-isopropyl-3,5-dibromothyronine ($IpBr_2$), or 3'-isopropyl-3,5-dimethylthyronine (Dimit) prebound, were produced by vapour diffusion at ambient temperature from 15% 2-methyl-2,4-pentanediol (MPD) as previously described (McGrath *et al.* 1994). The unique composition of the thyroid hormone permitted a phasing strategy in which cocrystals of the rTRα_1 LBD containing hormone analogs that differ at the 3,5, and 3' positions (T_3, $IpBr_2$, and Dimit) provided isomorphous derivatives. In this set of analogs, the halogen substituents (2Br and 3I atoms) function as heavy atoms, while the Dimit cocrystal acts as the parent. The initial 2.5Å multiple isomorphous replacement/anomalous scattering/density modified electron density map allowed the LBD to be traced from skeletons created in the molecular graphics program O (Jones *et al.* 1991). A model of the LBD was built in four fragments, Arg157-Gly182,

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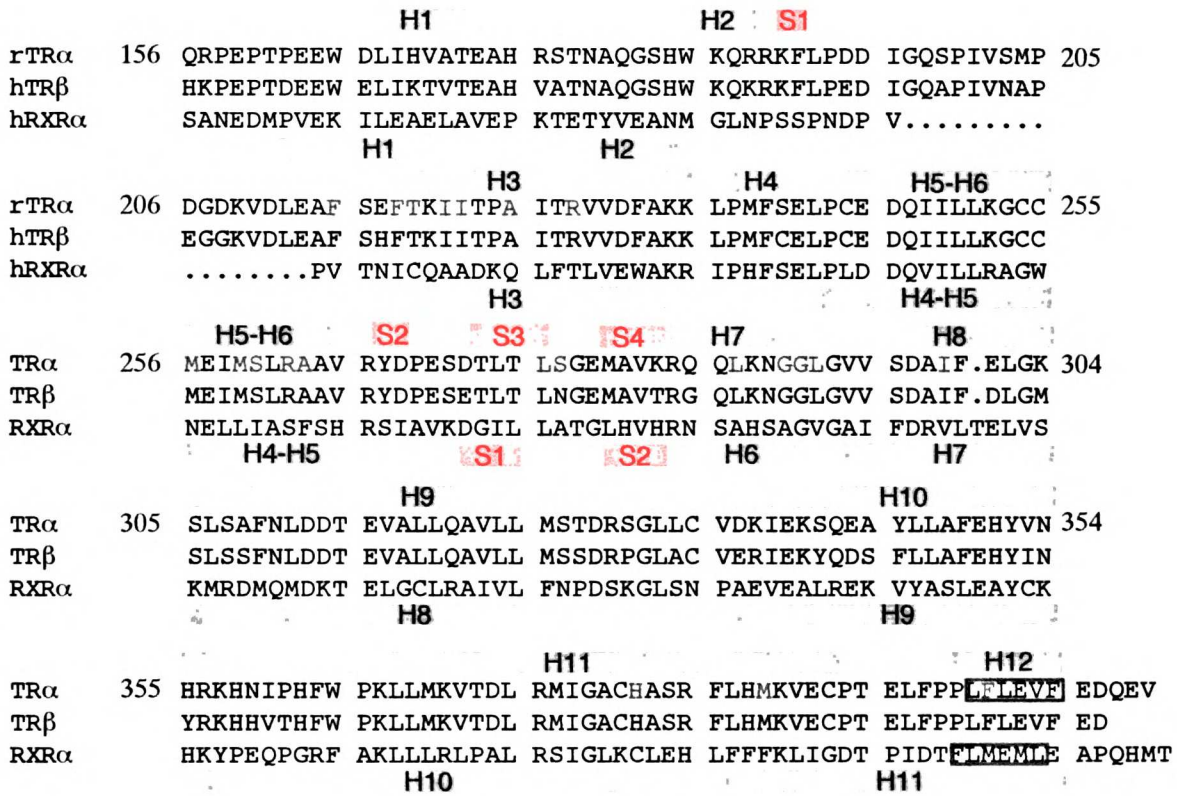
Figure 1-1 Comparison of the sequences for the rat α_1 and human ß thyroid hormone receptor ligand binding domains with the sequence for human RXRα LBD

The amino acid sequences of the rα$_1$TR LBD and hRXRα LBD are 21% identical and 47% similar. The secondary structure elements observed for rat α$_1$TR LBD and the hRXRα are shown above the sequence: α-helices are designated in blue, ß-strands in orange. Residues which contact the hormone are shown in purple type. The motif implicated in transcriptional activation, ΦΦXEΦΦ, where Φ represents a hydrophobic residue, is boxed.

Figure 1-1 Comparison of the sequences for the rat α_1 and human ß thyroid hormone receptor ligand binding domains with the sequence for human RXRα LBD



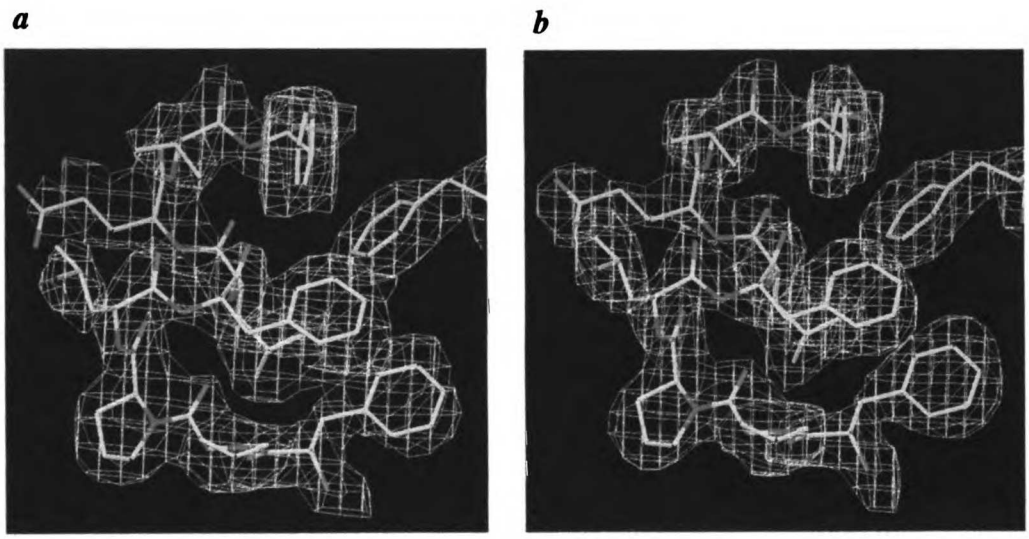
Trp185-Gly197, Ser199-Pro205, and Val210-Phe405, and refined in XPLOR using positional refinement and simulated annealing protocols (Brunger *et al.* 1987). Missing residues were built with the aid of difference density, except His184, Gln198, and Gly207, which could not be modeled. The final model was refined to R_{cryst} = 21.8% and R_{free} = 24.4% for data from 5.0 to 2.2Å (Figure 1.2; Table 1.1).

Figure 1-2 Experimental and final electron density for the C-terminal activation domain

(a) The experimental, MIRAS/density modified electron density at 2.5Å, contoured at 1σ. The depicted region is the C-terminal activation domain, residues Pro399-Phe405. The refined model, shown as a stick representation, is superimposed.

(b) The corresponding 2Fo - Fc electron density at 2.2Å, contoured at 1σ.

Figure 1-2 Experimental and final electron density for the C-terminal activation domain



COTTON

Table 1-1 Data collection, phasing and refinement statistics for the rat α_1TR LBD

	DIMIT	rTRα_1 LBD with T_3	$IpBr_2$
Data collection			
Cell dimensions			
a (Å)	117.16	117.19	117.18
b (Å)	80.52	80.20	80.12
c (Å)	63.21	63.23	63.13
β (°)	120.58	120.60	120.69
Resolution (Å)	2.2	2.0	2.1
Obs. Reflections, (no.)	57031	64424	66877
Unique Reflections, (no.)	22327	21023	23966
Completeness, (%)	87.0	82.4	93.7
*R_{sym} (%)	3.9	3.5	4.5
Phasing (15.0 - 2.5Å)			
†R_{der} (%)	-	19.6	11.6
No. of sites	-	3	2
‡Occupancy	-	44.6 (19.8)	35.0
(Anomalous)	-	50.2 (23.7)	35.0
	-	39.2 (22.3)	
§F_H/E			
centric (acentric)			
15.0-5.0 Å	-	3.67 (4.61)	2.25 (3.09)
5.0-3.0 Å	-	2.23 (2.75)	1.25 (1.85)
3.0-2.5 Å	-	1.64 (1.99)	1.15 (1.57)
‖R_{Cullis} (%)			
15.0-5.0 Å	-	33	44
5.0-3.0 Å	-	45	63
3.0-2.5 Å	-	60	65
Mean figure of merit	0.62	-	-

Refinement (5.0-2.2Å)

R_{cryst} (%)	21.8
R_{free} (%)	24.4
Rms bond length (Å)	0.006
Rms bond angle (°)	1.245

*$R_{sym} = 100 \times \Sigma_{hkl} \Sigma_i |I_i - I| / \Sigma_{hkl} \Sigma_i I_i$

†$R_{der} = 100 \times \Sigma_{hkl} |F_{PH} - F_H| / \Sigma_{hkl} |F_P|$

‡ The occupancy for the two bromine sites is set to 35 electrons. The occupancies of the iodine sites are relative to this value.

§Phasing power = $\langle F_H \rangle / \langle \varepsilon \rangle$, where $\langle F_H \rangle$ is the mean calculated heavy atom structure factor amplitude and $\langle \varepsilon \rangle$ is the mean estimated lack of closure.

||Rcullis = $\langle \varepsilon \rangle / \langle iso \rangle$, where $\langle \varepsilon \rangle$ is the mean estimated lack of closure and $\langle iso \rangle$ is the isomorphous difference.

¶$R_{cryst} = 100 \times \Sigma_{hkl} |F_o - F_c| / \Sigma_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes (for data $F/\sigma > 2$). The Rfree was calculated using 3% of the data, chosen randomly, and omitted from the refinement.

Architecture of TR LBD

The rTRα_1 LBD consists of a single structural domain packed in three layers, composed of twelve α-helices, H1-12, and four short β-strands, S1-4, forming a mixed β-sheet (Figure 1.3). The buried hormone and three antiparallel α-helices, H5-6, H9, and H10, form the central layer of the domain (Figure 1.4). H1, H2, H3, H4 and S1 form one face of the LBD, with the opposite face formed by H7, H8, H11, and H12. The predominantly helical composition and the layered arrangement of secondary structure is identical to that of the unliganded hRXRα, confirming the existence of a common nuclear receptor fold suggested by sequence analysis (Laudet, Hanni et al. 1992).

The first 35 amino acids of the N-terminus (Met122-Gln156) are not visible in the electron density maps. Interestingly, amino acids corresponding to Met122 - Ile151 of the rTRα DBD make extensive contacts with the minor groove of the DNA in the structure of TR:RXR DBD heterodimer (Rastinejad, Perlmann et al. 1995). The five disordered amino acids (Arg152-Gln156), which reside between the last ordered residue of the TR DBD and the first ordered residue of the LBD likely represent the effective "hinge" that links the DBD and LBD in the intact receptor.

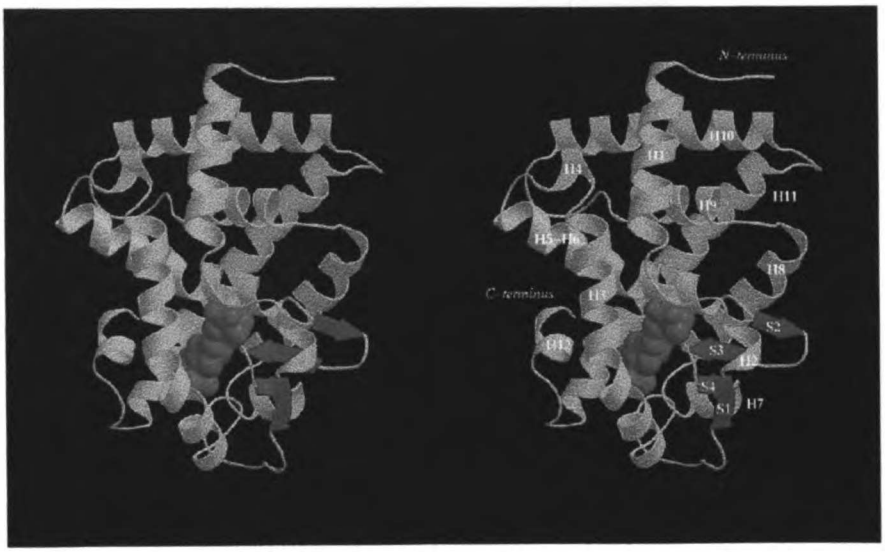
Figure 1-3 Stereoview of the rTRα_1 LBD

The hormone (magenta) is depicted as a space-filling model. α-helices and coil conformations are yellow, ß-strands are blue. Secondary structure assignments were obtained using the Kabsch and Sander algorithm as implemented in PROCHECK (Laskowski *et al.* 1993).

Figure 1.4 The hydrophobic core of the rTR α_1 LBD

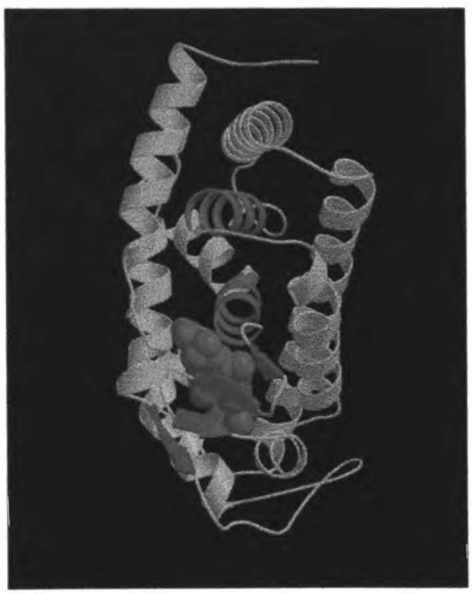
Ribbon drawing of the rTRα_1 LBD, showing the three layers of the domain. The hydrophobic core of the receptor is formed by two α-helices and the hormone. The hormone is depicted as a space-filling model. Core-forming elements are shown in magenta.

Figure 1-3 Stereoview of the rTRα_1 LBD



Fig

Figure 1-4 The hydrophobic core of the rTRα_1 LBD



The TR LBD is visible beginning at Arg157, and continues in an extended coil to the start of H1. A turn of α-helix, H2, covers the hormone binding cavity. Immediately following, a short β-strand, S1, forms the edge of the mixed sheet, parallel to S4, the outermost of the three antiparallel strands. The chain is mostly irregular until H3 begins, antiparallel to H1. H3 bends at Ile221 and Ile222, which contact the ligand. The chain turns almost 90° at the end of H3 to form an incomplete α-helix, H4. The first buried core helix, H5-H6, follows, its axis altered by a kink near the ligand at Gly 253. The helix is composed of mostly hydrophobic sidechains, interrupted by two striking exceptions: Arg262 is solvent inaccessible and interacts with the ligand carboxylate, and Glu257 meets Arg329 from H9 and Arg375 from H11 in a polar invagination. H5-H6 terminates in a short β-strand, S2, of the four strand mixed sheet. S3 and S4 are joined through a left-handed turn, and further linked by a salt bridge between Lys283 and Asp272. Following S4, H7 and H8 form an L, stabilized by a salt bridge between Lys288 and Asp297. The turn between H7 and H8 adopts an unusual conformation, a result of interaction with ligand and its glycine rich sequence. The second core helix, H9, is antiparallel to the neighboring H5-H6, and contains two buried polar side chains, Glu315 and Gln320. Glu315 forms a buried salt bridge with His358 and Arg356. The oxygen of Gln320 forms a hydrogen bond with the buried sidechain of His175. The chain then switches back again to form H10, also antiparallel to H9. H11 extends diagonally across the full length of the molecule. Immediately after H11, the chain forms a type II turn, at approximately 90° to H11. The chain then turns again to form H12, which packs loosely against H3 and H11as part of the hormone binding cavity. The five C-terminal amino acids, Glu406 -Val410, are disordered.

The hormone binding cavity

Dimit, an isostere of T_3, is a thyroid hormone agonist (Jorgenson 1978). Therefore, the conformation of the Dimit-bound receptor should resemble the T_3-bound conformation. The ligand is buried within the receptor, providing the hydrophobic core for a subdomain of the protein (Figure 1.5) ; H5-H6 and H9 complete the bipartite core of the receptor. An extensive binding cavity is constructed from several structural elements. The cavity is enclosed from above by H5-H6 (Met 256 - Arg266), from below by H7 and H8 and the intervening loop (Leu287 - Ile299), and along the sides by H2 (Trp185- Gln187), by the turn between S3 and S4 (Leu276-Ser277), by H3 (Phe215-Arg228), by H11 (His381-Met388) and by H12 (Phe401-Phe405). The volume of the cavity, calculated by GRASP (Nicholls *et al.* 1991)(600 $Å^3$), is essentially the volume of the hormone (530 $Å^3$). The remaining volume is occupied by water molecules surrounding the amino-propionic acid substituent. Figures 1.6 and 1.7 depict the contacts between the LBD and the ligand.

Figure 1-5 The hormone is completely buried within the receptor

A cross-sections of a space-filling model of the rTR α_1 exposes the hormone (magenta), tightly packed within the receptor. The view is that of Figure 1.4.

Figure 1-6 Stereoview of the hormone binding cavity

Residues which interact with the ligand are shown as side chains. The coloring scheme reflects the chemistry of the side chain: basic residues (argine and histidine) are blue, aromatic hydrophobics (phenylalanine) are brown, aliphatic (unbranched) hydrophobics (alanine and leucine) are forest green, aliphatic (branched) hydrophobics (isoleucine) are light green, hydrophilic residues (serine and threonine) are dark red, and sulfur-containing residues (methionine) are dark goldenrod. The ligand is colored magenta. View is identical to that of Figure 1.4. The residues are (clockwise from top): Arg 262; Ala 263; Ser 277; Leu 292 (left); Leu 276 (right); Ile 221; Phe 218; Phe 215; Thr 219; Met 388; Ile 222; Phe 401; Phe 405; His 381; Ala 225; Met 256 (left); Met 259 (right); Arg 228.

Figure 1-5 The hormone is completely buried

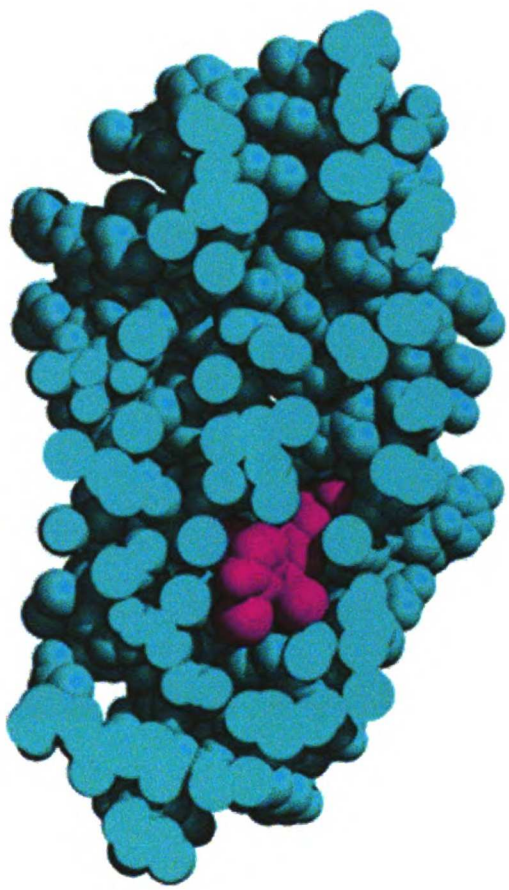
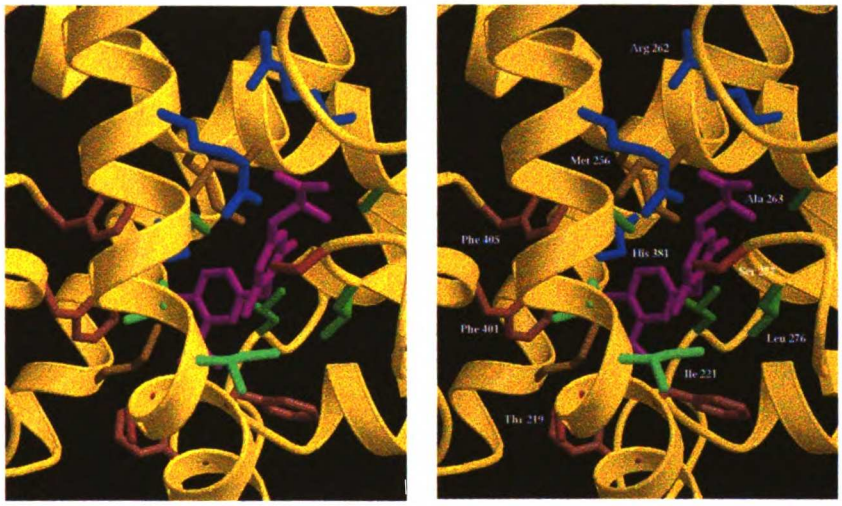


Figure 1-6 Stereoview of the hormone binding cavity



Figure

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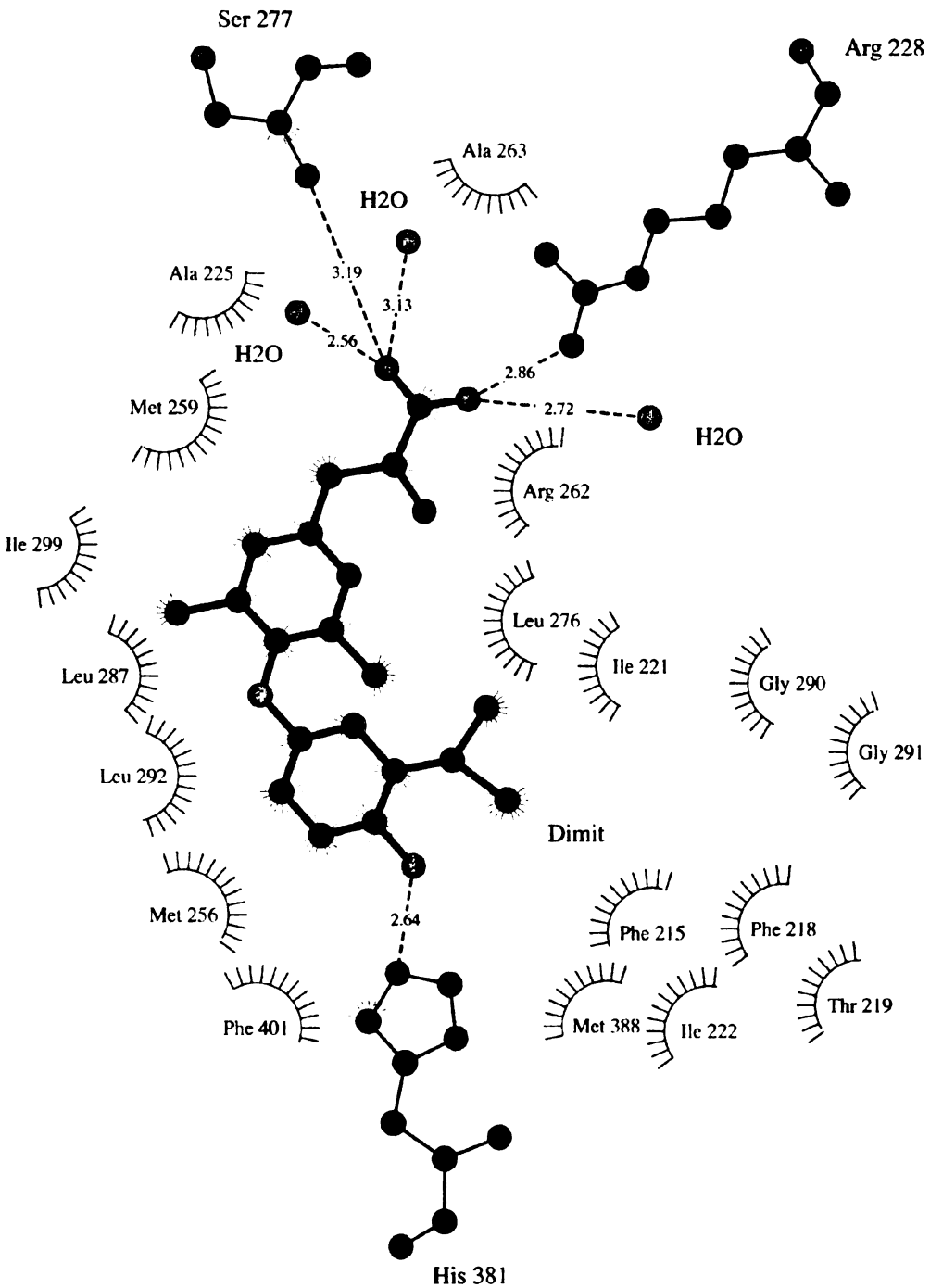
Figure 1-7 Schematic of the hormone binding cavity

Residues which interact with hormone appear at approximately the site of interaction. Hydrogen bonds are shown as dashed lines between the bonding partners; distances for each bond are listed. Nonbonded contacts are shown as radial spokes, which face toward interacting atoms. Only three direct hydrogen bonds are made: from Arg228 and Ser277 to the carboxylate group, and from His381 to phenolic hydroxyl. The ether oxygen is found in a hydrophobic environment, surrounded by Phe218, Leu287, Leu276 and Leu292. Contacts to the inner ring are provided by the following side chains: Ile221, Ile222, Ile229 and Ala225 from H3; Met259, Ser260, Arg262, and Ala263 from H5; and Leu276 from the loop between S3 and S4. Ser260 and Ile299; and Phe218, Ile221, and Ile222 form pockets for the 3- and 5-methyl substituents, respectively. Contacts to the outer ring are provided by three phenylalanines in plane-perpendicular fashion: Phe215, Phe218, and Phe401. Other side chain contacts are made by Gly290-291 and Leu292 from the loop between H7 and H8, His 381 and Met 388 from H11, and Ile222 and Thr219 from H3. Gly290 and Gly291 form a pocket for the 3'-isopropyl substituent. A subset of these interactions are described in the text. Figure was prepared using Ligplot (Wallace *et al.* 1995).

Figure 1-7 Schematic of the hormone binding cavity



The inner and outer rings of the ligand are nearly perpendicular, with the 3' substituent of the outer ring projecting down and away from the inner ring, as predicted by studies of receptor binding to conformationally restricted analogs (Jorgensen 1978). The amino-propionic acid and the outer phenolic ring assume the transoid conformation, each on opposite sides of the inner ring. The torsion angle χ_1 for the amino propionic acid is 300°.

The amino-propionic acid substituent is packed loosely in a polar pocket formed by side chains from H2, H4 and S3. The carboxylate group forms direct hydrogen bonds with the guanidium group of Arg 228 and the amino nitrogen of Ser 277. In addition, Arg262, Arg266 and Asn179 interact with the carboxylate through water-mediated hydrogen bonds. No hydrogen bond is formed by the amino nitrogen. The interactions of the amino-propionic acid substituent are consistent with the fact that 3,5-diiodo-3'-isopropylthyroacetic acid, or triac, which lacks the amino nitrogen, has a binding affinity equal to that of T_3, suggesting that the amino nitrogen and longer aliphatic chain of T_3 do not contribute greatly to binding affinity (Apriletti, Baxter et al. 1995).

The diphenyl ether, in contrast, is buried within the hydrophobic core. The inner ring packs in a hydrophobic pocket formed by H3, H5-6, and S3. Pockets for the 3- and 5-methyl substituents are not completely filled, as expected since the van der Waals radius of methyl substituent for Dimit is smaller than the iodine substituent provided by the thyroid hormone T_3.

The outer ring is packed tightly in a pocket formed by H3, H5-6, H7, H8, H11 and H12, and the loop between H7 and H8. The ether oxygen is in a hydrophobic environment defined by Phe218, Leu287, Leu276, and Leu292. The absence of a hydrogen bond to the ether oxygen is consistent with its proposed role in establishing the correct stereochemistry of the phenyl rings, as suggested by potent binding of hormone analogs with structurally similar linkages possessing reduced or negligible hydrogen bonding capability (Jorgensen

1978). The 3'-isopropyl substituent contacts Gly290 and Gly291. The structure of the pocket may explain why activity is highest for a 3'-isopropyl substituent, and decreases with added bulk (Jorgensen 1978). The only hydrogen bond in the hydrophobic cavity is formed between the phenolic hydroxyl and His381 Nε2. The conformation of His381 is stabilized by packing contacts provided by Phe405, and Met256.

The presence of a 5' substituent larger than hydrogen decreases the binding affinity for hormone. The more abundant thyroid hormone, 3,5,3',5'-tetraiodo-L-thyronine (T_4), contains an iodine at this position, and binds the receptor with 2% of the affinity of T_3. The structure suggests that discrimination against T_4 is accomplished through the combination of steric conflict by Met256 and possibly the constraints imposed by the geometry of the hydrogen bond from His381 to the phenolic hydroxyl.

Deletion and antibody competition studies suggest the involvement of residues Pro162 to Val202 in ligand binding (Lin *et al.* 1991; Bhat *et al.* 1995). The region does not directly contact hormone in the bound structure, although H2 packs against residues forming the pocket that interacts with the amino-propionic acid group. H2, then, may stabilize these residues in the bound state. H2, with β-strands S3 and S4, might also represent a prevalent entry point for ligand. Studies of receptor binding to T_3 affinity matrices demonstrate that only a linkage to the amino-propionic acid is tolerated, suggesting that steric hindrance present in other linkages prevent binding (Latham *et al.* 1981). Furthermore, the crystallographic temperature factors suggest the coil and β-strand region is the most flexible part of the domain (data not shown). Participation of this region, part of the Hinge domain between the DBD and LBD, in binding hormone may provide structural means for ligand binding to influence DNA binding, since parts of the Hinge domain contact DNA (Tsai and O'Malley 1994; Rastinejad, Perlmann et al. 1995).

Distribution of Mutations in Generalized Resistance to Thyroid Hormone Syndrome

The syndrome Generalized Resistance to Thyroid Hormone (GRTH) is characterized by high serum levels of $T_3$and T_4 with non-suppressed levels of thyroid stimulating hormone (TSH), and a generally euthyroid state (Refetoff *et al.* 1993). Genes from these patients contain mutations in the TR β_1 LBD, clustered within two regions in the linear sequence. The mutant receptors exhibit reduced to undetectable hormone binding activity, and typically antagonize the activity of wild-type receptors. The antagonism can be overcome in many cases by elevated concentrations of hormone which compensate for the mutant receptors' reduced affinity. Since the rTRα_1 LBD shares 88% sequence identity with the hTRβ LBD, the bound structure can serve as a model for study of the GRTH mutations.

GRTH mutations cluster into two regions of secondary structure: S4, H6, H7 and the connecting loop with H8 (cluster 1); and H11, and H12 (cluster 2). Provocatively, the mutations describe the subdomain of the receptor for which hormone provides the hydrophobic core (Figure 1.8). The apparent pattern confirms the central effect of GRTH on hormone binding and hormone-dependent processes. The mutations appear to influence ligand binding by either disrupting direct contacts, or altering interactions that stabilize the bound conformation. Furthermore, the mutations would be expected to vary in their

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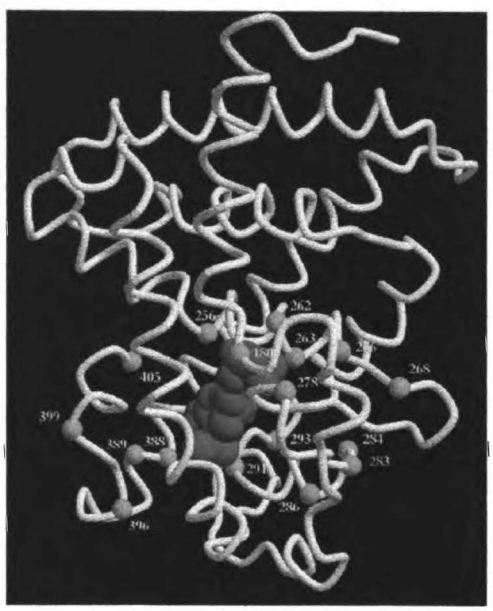
Figure 1-8 Selected mutations associated with GRTH mapped onto the rTRα_1 LBD

The positions of the residues are shown as blue spheres. The mutated residues are: A180T, M256T, R262H, A263T, R267C,D268H, G278R, Δ283T, R284W, Q286H, G291R, G293E, R384H, M388V, K389E, L396H, P399T, F405C (Beck-Peccoz *et al.* 1994).

Figure 1-8 Selected mutations associated with GRTH mapped onto the rTRα_1 LBD



quantitative effect on ligand binding, and also in the ability of ligand to convert the mutated receptor to the active conformation. Moreover, receptor activities retained by the mutant receptors, such as dimerization, localize outside the GRTH cluster, and are probably unaffected (Selmi and Samuels 1991; Au-Fliegner *et al.* 1993).

Dimerization and Heterodimerization

The TR is capable of both homodimerization, and heterodimerization with RXR, on appropriate DNA response elements (Glass 1994; Ribeiro, Kushner et al. 1995). Several mutations which affect TR dimerization involve a heptad repeat of hydrophobic residues (Leu367-Leu374), that is proposed to form a protein interface similar to a leucine zipper (Forman and Samuels 1990; Selmi and Samuels 1991; Au-Fliegner, Helmer et al. 1993).

The rTRα_1 LBD constructs the crystal lattice as a monomer. The limited surface area buried by the intermolecular contacts between monomers suggests the contacts do not represent a physiological dimer association. The heptad repeat is located at the N-terminal portion of H11. Many of the residues implicated in dimerization are found on the surface of H11, forming an extensive hydrophobic patch. In fact, the face of the rTRα_1 LBD formed by H10 and H11 is composed mainly of hydrophobic residues, with alternating concave and convex surface, and appears suited to protein interaction. In the hRXRα, the N-terminal portion of H10, together with H8 and H9, does indeed form a dimerization surface (Bourguet, Ruff et al. 1995).

Transcriptional Activation Domains

The C-terminal 17 amino acids of the TR, referred to as the C-terminal activation domain, are implicated in hormone-dependent transcriptional activation. Mutations of key residues within the domain decrease or abolish ligand-dependent activation. (Zenke *et al.* 1990; Saatcioglu *et al.* 1993; Barettino, Vivanco Ruiz et al. 1994; Tone, Collingwood et al. 1994; Baniahmad *et al.* 1995) Furthermore, the domain functions autonomously as a transcriptional activator when fused to an unrelated DBD (Barettino, Vivanco Ruiz et al. 1994; Tone, Collingwood et al. 1994; Baniahmad, Leng et al. 1995). Thus, the region may represent a discrete functional domain, which in the receptor is regulated by hormone. Deletions or mutations of this domain also affect affinity for hormone, further evidence that ligand binding and activation are linked. Similar activation domains have been identified in the other nuclear receptors (Danielian, White et al. 1992; Durand, Saunders et al. 1994; Leng *et al.* 1995). The domain is proposed to form an amphipathic helix, within which the highly conserved motif ΦΦXEΦΦ (where Φ represents a hydrophobic residue) is proposed to mediate interactions between the receptors and transcriptional coactivators (Zenke, Munoz et al. 1990; Barettino, Vivanco Ruiz et al. 1994). Recently, several proteins were identified which bind the TR, or other nuclear receptors, in a ligand-dependent manner through the C-terminal domain (Cavailles *et al.* 1995; Le Douarin *et al.* 1995; Lee *et al.* 1995; Lee *et al.* 1995) (D.D. Moore, personal communication).

The C-terminal activation domain of the TR forms an amphipathic helix, H12, which nestles loosely against the receptor to form part of the hormone binding cavity. The helix packs with the hydrophobic residues facing inward towards the hormone binding cavity, and the charged residues, including the highly-conserved glutamate, extending into the solvent (Figure 1.9*a*). Phe401 interacts directly with the hormone, forming a van der Waals contact with the plane of the outer phenyl ring. Phe405 interacts with His381, perhaps stabilizing its hydrogen bonding conformation. Participation of Phe401 and Phe405 in binding hormone explains how mutation of these residues decreases hormone binding affinity (Barettino, Vivanco Ruiz et al. 1994). Furthermore, the impact of these mutations on activation likely derives from a role in stabilizing the domain in the bound structure. Glu403 extends into the solvent as an ordered residue, consistent with its critical role in transactivation and presumed interactions with activator proteins (Figure 1.9*b*). The other charged residues, Glu406 and Asp407 are disordered.

Two other sequences in the TR, τ2 and τ3, activate transcription when expressed as fusion proteins with a DNA-binding domain (Baniahmad, Leng et al. 1995). The sequences, discovered in the TRβ, correspond to TRα residues Pro158-Ile168 in H1 (τ2), and Gly290-Leu319 in H8 and H9 (τ3). Unlike the C-terminal activation domain, τ2 and τ3 do not appear to represent modular structural units in the rTRα_1 LBD, nor present a surface for protein-protein interactions: the critical aspartate/glutamate residues of τ3 are

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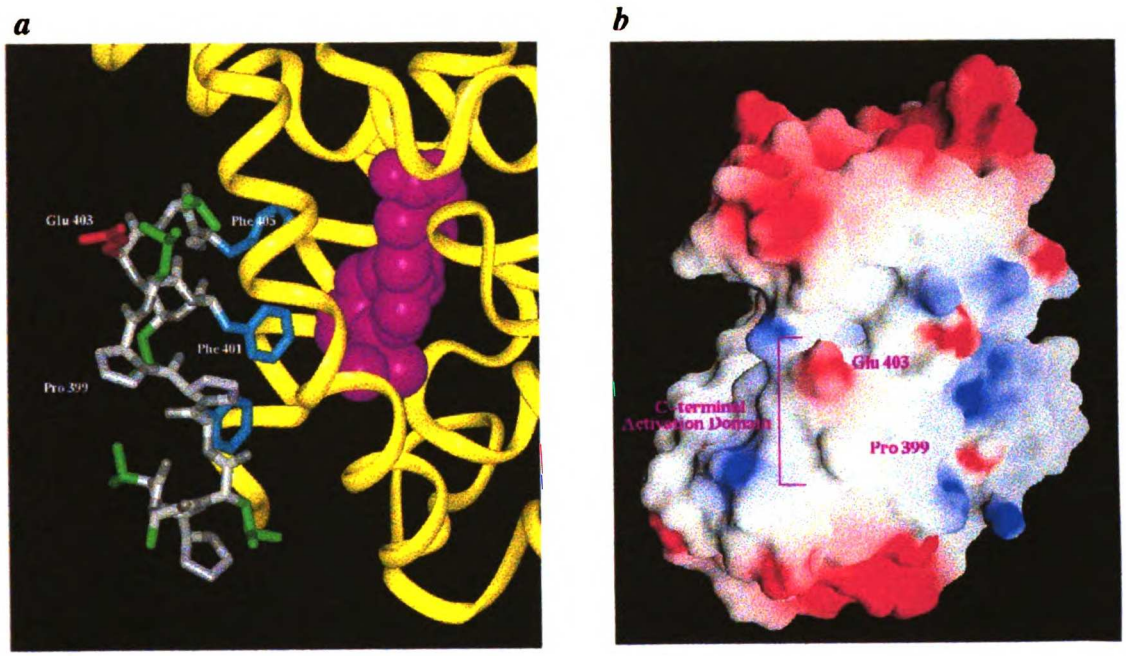
Figure 1-9 The C-terminal activation domain

(a) The view is identical to that of Figure 1.2. Residues which comprise the C-terminal activation domain (Pro393-Phe405) are depicted as a stick representation. The domain forms an amphipathic helix, beginning at Pro399 (grey). Hydrophobic residues, particularly Phe401 and Phe405 (blue), face inward towards the hormone. Glu403 (red) projects outward into the solvent. Side chains of other non-proline residues are green.

(b) Electrostatic potential surface of the C-terminal activation domain, calculated using GRASP (Nicholls, Sharp et al. 1991). Negative electostatic potential is red, positive electrostatic potential is blue. The C-terminal activation domain forms a largely hydrophobic surface, with the critical residue Glu403 presented as a singular patch of negative charge.

Figure 1-9 The C-terminal activation domain



located on two separate helices, and do not form a single surface; the charged residues of τ2 are engaged in ion pair interactions within the LBD. Thus, τ2 and τ3 may not function as activation domains in the context of the entire receptor.

Hormone-Induced Conformational Change

Plasma proteins bind hormone without undergoing a conformational change through a static binding pocket formed between monomers or domains. For example, the tetrameric thyroid-binding plasma protein transthyretin forms a solvent-accessible hormone-binding channel at the oligomer interface (Blake and Oatley 1977; Blake *et al.* 1978). The structure of the protein is unchanged upon binding hormone (Blake and Oatley 1977; Blake, Geisow et al. 1978). By contrast, the structural role for ligand in the bound rTRα_1 LBD predicts the receptor would differ in the bound and unbound states. In the absence of hormone, the receptor would possess a cavity at its core, uncharacteristic of a globular protein. Hormone binding is a dynamic process, which completes the hydrophobic core of the active receptor, and induces an altered conformation(Tsai and O'Malley 1994; Ribeiro, Apriletti et al. 1995; Ribeiro, Kushner et al. 1995).

An exact description of the hormone-induced conformational changes requires comparison of the structures of the liganded and the unliganded TR. The structure of the unliganded hRXRα may substitute as a model for the unliganded TR (Bourguet, Ruff et al. 1995). The rTRα_1 LBD and hRXRα LBDs adopt a similar fold, and it is possible that the structural similarity extends to the conformational changes.

There are three major differences between the two structures, which indeed appear related to hormone binding. First, the bound rTRα_1 LBD structure is more compact, with the hormone tightly packed within the core of the receptor. By contrast, the unliganded hRXRα LBD contains several internal hydrophobic cavities. The presence of such cavities

is unusual in folded proteins. Two of these cavities were proposed as possible binding sites for 9-cis retinoic acid, though these sites only partly overlap with the single binding cavity observed in the liganded rTRα_1 LBD. The second difference involves H11 in the rTRα_1 LBD, which contributes to the hormone binding cavity. H11, continuous in the rTRα_1 LBD, is broken at Cys 432 in the RXR, forming a loop between H10 and H11 in the hRXRα. This residue corresponds to His381 in the TR, which provides a hydrogen bond to the outer ring hydroxyl of the ligand. Furthermore, the hormone binding cavity occupied by ligand in the rTRα_1 LBD is interrupted in the hRXRα by the same loop, forming an isolated hydrophobic pocket in the RXR with H6 and H7. In the bound rTRα_1 LBD, the corresponding helices H7 and H8 are contiguous with the binding pocket, and enclose the hormone binding cavity from below.

The third difference between the two receptors is the position of the C-terminal activation domain. While the C-terminal activation domain forms α-helices in both receptors, the domain in the rTRα_1 LBD follows a proline-rich turn, and lies against the receptor to contribute part of the binding cavity. In contrast, the activation domain in the unliganded hRXRα is part of a longer helix which projects into the solvent.

These differences suggest a model for an alternate conformation of the TR LBD assumed in the absence of ligand. Perhaps in the unliganded TR, the subdomain of the receptor surrounding the hormone binding cavity is loosely packed, with the binding cavity occluded by a partly unstructured H11, providing a substitute core for the receptor. Upon binding hormone, residues which form a coil in the unbound receptor may engage the hormone, and continue H11. The ordering of H11 could unblock the hydrophobic cavity, allowing H7 and H8 to interact with hormone. The extended hydrophobic cavity then collapses around the hormone, generating the compact bound structure.

It is difficult to predict the precise ligand-induced conformational changes in the C-terminal activation domain. Although the domain might assume an extended structure in the unliganded TR, and repack upon ligand binding, the change is probably more subtle, since the amino acid sequence of the rTRα_1 in the turn (393-PTELFPP-399) significantly reduces its propensity to form an α-helix. In fact, it is remarkable that there is a continuous α-helix in the hRXRα, given the rather low helical propensity of the corresponding hRXRα sequence (444-DTPIDT-449).

Nevertheless, it is likely that the conformation of the C-terminal activation domain in the bound structure differs from that in the unbound receptor. The packing density , defined as the number of atoms within 4.5Å, is 1.5σ below the mean for the activation domain as compared with the entire LBD, suggesting the domain packs loosely even in the bound structure. Moreover, the majority of packing contacts for the C-terminal domain in the bound receptor are provided either by residues which interact with ligand, such as His381, or by the ligand itself. Without the stabilization provided by hormone, it is likely that the C-terminal activation domain is disordered prior to hormone binding. The hormone-induced changes which affect H11 and H12 are reinforcing, and lead to the formation of the active conformation.

Mechanisms of Hormone-dependent Regulation

In mammalian cells, the TR commonly exhibits two activities: transcriptional repression in the unliganded state, and transcriptional activation in the liganded state (Ribeiro, Apriletti et al. 1995). A model for TR function proposes that these activities are mediated through hormone-dependent interactions with other proteins (Baniahmad, Leng et al. 1995). The unliganded TR is thought to bind a corepressor protein involved in repression of transcription (Chen and Evans 1995; Horlein *et al.* 1995). Ligand binding appears to induce both a dissociation of this protein with a relief of repression, and an association with

another protein(s) that participates in the activation of transcription. Deletion of part of the C-terminal activation domain (Leu402-Val410), abolishes the ligand's influence on both activities; the truncated receptors bind ligand (though at a lower affinity), and undergo certain conformational changes in response to ligand binding, but are constitutive repressors (Baniahmad, Leng et al. 1995). These observations imply that only a subset of the conformational changes in the receptor that occur upon hormone binding influence the regulation of transcription by the receptor.

Ligand-induced changes in the conformation of the C-terminal activation domain, as discussed above, could influence its ability to interact with other proteins involved in mediating transcriptional regulation by the receptor. Inspection of the structure suggests that the partial deletion of the C-terminal activation domain would not compromise the structural integrity of the hormone-binding cavity, or prevent the formation of the compact bound state. An increased affinity of the C-terminal activation domain for other proteins could result from ordering of the domain, to present a more stable, and probably more extensive, interaction surface in the bound TR. Alternatively, ligand binding may release the domain from interactions within the LBD which obscure residues important for coactivator binding.

Ligand-induced changes can affect other receptor activities, such as dimerization. For example, hormone binding by the TR can modestly affect dimerization in a response-element specific fashion (Ribeiro *et al.* 1992; Bendik and Pfahl 1995). The hRXRα forms a dimer through a surface provided by the N-terminal part of H10, together with H8 and H9 (Bourguet, Ruff et al. 1995). Examination of rTRα_1 LBD suggests a similar surface might mediate TR dimerization. However, critical contacts with hormone are contributed by residues in the C-terminus of H11. The direct participation of H11 in both processes is reminiscent of a similar colocalization of ligand-binding and dimerization in the estrogen receptor, and suggestive of a central role for hormone in altering the dimerization surface

upon ligand binding (Fawell *et al.* 1990). Such an alteration could be accomplished through a bend or rotation of H10, as may occur in the ordering and extension of H11 described above.

CONCLUSION

The structural role for ligand revealed by the bound rTRα_1 LBD is fundamental to the mechanism of biological regulation of the receptor. Hormone controls receptor function through an absolute requirement for hormone in creating the active conformation of the receptor. Through a dynamic process, hormone completes the hydrophobic core of the active conformation of the receptor, and directs the alignment of secondary structure elements critical for receptor function. We speculate that this process may resemble the registration of already formed secondary structure elements that occurs late in protein folding.

Given the sequence conservation within the LBD between members of the nuclear receptor superfamily, and the demonstrated structural similarity, we suggest the binding mode observed here will be the prototype for the nuclear receptors. While the mechanisms through which hormone exercises control over receptor function may differ between the nuclear receptors, the structural role for ligand, and its attendant conformational changes, will underlie these mechanisms. The implications of the structural role for ligand may explain the action of an entire class of hormones and receptors.

MATERIALS AND METHODS

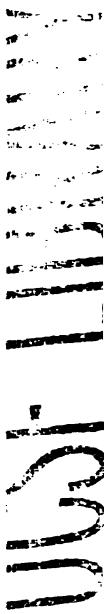
Purification and expression Rat α_1TR LBD, residues Met122 - Val410, was purified from *E.coli* as described (Apriletti, Baxter et al. 1995). Material from a single preparation was divided into batches, and quantitatively bound with either Dimit, T_3, or $IpBr_2$ for the final purification step. Diffraction quality crystals (dimension 0.5 X 0.2 X 0.075 mm^3) were grown at ambient temperature from hanging drops containing 15% 2-methyl-2,4-pentanediol (MPD) and 8.7 (Dimit), 5.5 ($IpBr_2$), or 2.3 (T_3) mg ml^{-1} of protein as described (McGrath, Wagner et al. 1994). The crystals are of the monoclinic space group C2, with one monomer in the asymmetric unit.

Data collection Data from each of three cocrystals were measured on a Mar area detector at Stanford Synchrotron Radiation Laboratory beamline 7-1 (λ = 1.08 Å) using 1.2° oscillations; data from the T_3 cocrystal were measured with the b* axis approximately parallel with the spindle. The crystals were flash frozen at -178°C in a nitrogen gas stream with the MPD mother liquor serving as the cryosolvent. An orientation matrix for each crystal was determined using REFIX (Kabsch 1993). Reflections were integrated with DENZO, and scaled with SCALEPACK (Otwinowski and Minor 1997). For the T_3 data set, Bijvoet pairs were kept separate, and locally scaled using MADSYS (W. Hendrickson and W. Weis).

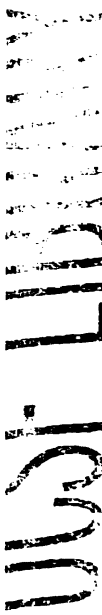
Phasing Cocrystals prepared from the three isosteric ligands were isomorphous. MIR analysis was performed using programs from the CCP4 suite (Project 1994) . Difference Pattersons were calculated for both T_3 and $IpBr_2$, taking the Dimit cocrystal as the parent. The positions of the three iodine atoms in the T_3 difference Patterson were unambiguously determined from the Harker section of the density map as peaks of 11σ above background. The positions for the two bromine atoms in the $IpBr_2$,were located independently, as peaks 8σ above the noise level. Phases for the $rTR\alpha_1$ LBD were calculated from the solution to the $IpBr_2$ difference Patterson, and used to confirm the location of the unique third iodine of the T_3 cocrystal. Halogen positions were refined with MLPHARE, including the anomalous contributions from the iodine atoms (Otwinowski 1991). The MIRAS phases were improved through solvent flattening/histogram matching using DM (Cowtan 1994).

Model building and refinement A model of the $rTR\alpha_1$ LBD with Dimit bound was built with the program O from the solvent flattened MIRAS 2.5Å electron density map (Jones, Zou et al. 1991). The initial model, without ligand, (R_{cryst} = 40.1%), was refined using least-squares protocols with XPLOR. The Dimit ligand was built into unambiguous Fo-Fc difference density during the following round. Subsequent refinement employed both least-squares and simulated annealing protocols with XPLOR (Brunger, Kuriyan et al. 1987). Individual atomic B-factors were refined isotropically. As defined in PROCHECK, all residues are in allowed main-chain torsion angle regions (Laskowski, Macarthur et al. 1993). The current model is missing 34 residues (Met122-Gln156) at the N-terminus, 5 residues (Glu406-Val410) at the C-terminus, and the individual residues His184, Gln198, and Gly207. In addition, the following residues were not modeled beyond Cβ due to poor density: 186, 190, 206, 209, 240, 302, 330, 337, 340, 343, 359, 395. The average B-value for protein atoms is 34.5Å^2. The final model consists of the $rTR\alpha_1$ LBD, residues Arg157-Ser183, Trp185-Gly197, Ser199-Asp206, and Asp208-Phe405; three cacodylate-modified cysteines: Cys334, Cys380, and Cys392; and 73

solvent molecules modeled as water (2003 atoms). Coordinates will be deposited with the Brookhaven Protein Databank.



Acknowledgments We thank H. Nguyen for help in receptor expression; M. Bolger for providing the $IpBr_2$ ligand; J. Buchbinder, S. Gillmor, and E. Sablin for help in synchrotron data collection; W. Weis for assistance with MADSYS; D. Freymann, P. Kushner, V. Ramalingam, T. Scanlan, and A. Shiau for discussion; S. Gillmor, C. Honchell, T. Mau, R. Ribeiro and K. Yamamoto for critical reading of the manuscript. We also acknowledge the following beamlines, and their staff: Photon Factory BL6A2, NSLS X4A, CHESS F1, and especially SSRL 7-1. This work was supported in part by grants from the N.I.H. (J.B. and R.J.F,) and the Programs of Excellence in Molecular Biology (R.J.F.). R.L.W. is supported by an NSF predoctoral fellowship.



AFTERWORD

The original text of this chapter was published simultaneously in *Nature* with "Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid," by J.P. Renaud, *et al.* (**378** (6558): 681-9). That paper presents the structure of the complex of the retinoic acid receptor LBD with its cognate ligand, retinoic acid, and contains a more quantitative comparison of the liganded RAR and unliganded RXR LBDs. Nevertheless, that analysis identifies the same global differences which we noted, the most significant being the two distinct positions of H12. In agreement with our conclusions, the authors argue that the positioning of H12 against the body of the receptor represents a fundamental conformational change which follows ligand binding, producing a molecular surface on the LBD competent for interaction with coactivator proteins, such as members of the p160 family.

Both papers suffer from their reliance on the structure of the RXR LBD as a surrogate for the unliganded state of their individual receptor LBDs. Certainly, RXR is not an ideal choice: in phylogenetic trees of the nuclear receptor superfamily generated from multiple sequence alignments, RXR is assigned to a separate subfamily than either TR or RAR, due in part to sequence differences that may support the peculiar structure of the RXR (such as P423, F437-439, and the divergence in the loop between H11 and H12 - motifs present in the presumably related receptors DAX1 and USP) (Laudet *et al.* 1992). Furthermore, its putative ligand, 9-cis retinoic acid, was likely acquired through convergent evolution, given the divergence between RARs and RXRs in those residues contacting retinoic acid; by contrast, the ligand contacts are almost absolutely conserved across species in the TR and RAR. Of the two papers, however, Renaud *et al.* is more guilty of forcing mechanistic identity upon structural similiarity between TR/RAR and RXR, the result being the

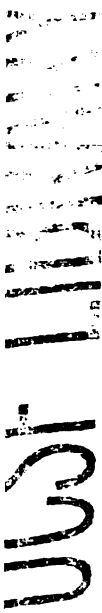
"mouse-trap" model of ligand activation (further elaborated in Moras and Gronemeyer 1998)

The "mouse trap" model appears to be a economic solution to the problems of ligand entry and ligand-dependent transcriptional activation: guided electrostatically, the ligand enters the ligand pocket through an opening created by the displaced H12, which reorients to seal the ligand in the interior of the receptor. Obviously, the "mouse trap" demands a similarly rigid, extended H12 in RAR to function as the snap; but the motif PGxxPP preceding H12 in RAR would favor a coil conformation rather than an extension of H12, as we argued in the case of the TR. The arguments for ligand entry through H12 to spring the trap are similiarly spurious. First, no "opening" to the ligand binding cavity created by displacement of H12 is observed in the unliganded RXR; in contrast, the structure of the polar pocket, which we suggested might provide a route for ligand entry, is quite different, with the charged residue R316 rotated into solvent to expose a solvent-accessible ligand cavity. Second, the electrostatic field lines which supposedly attract the ligand to the cavity, along a path passing through the displaced H12,were calculated for artificial unliganded state of RAR in which only the ligand and H12 were removed. The field calculation is therefore biased toward the liganded state; a more convincing example of electrostatic attraction would be the field calculation for the unliganded RXR, but rearrangement of the polar residues in RXR noted above does not provide the precise focusing claimed for RAR. Third, the primary interaction suggested to stabilize the bound state, a salt-bridge between K264 in H5-H6 and E414/E417 in H12, is both energetically insignificant in solution, and absent in the TR. Finally, the receptor must convert between bound and unbound states in equilibrium, but the conformational change required to release the ligand from the mouse-trap entails reexposing several hydrophobic residues on H12 to solvent. The polar pocket, however, allows the necessary dynamics, given the high thermal factors for the residues in that region; furthermore, the closed conformation seen in the liganded state is maintained only by a few hydrogen bonds.

Thus, if ligand does produce conformational changes in the receptor LBD that align H12 to promote coactivator binding, the exact nature of those changes are unlikely to involve the rigid rotation central to the "mouse-trap" model. A reported structure of the unliganded PPARγ LBD, in which H12 packs against the body of the receptor as observed in the liganded TR and RAR LBDs, suggests a more modest adjustment in H12 on ligand binding than that imagined by either Renaud *et al.* or ourselves. But the ability of ligand to produce structurally and functionally distinct conformations of the receptor was confirmed by the structures of ER LBD complexed with estrogen and with reloxifene, an AF-2 antagonist (Brzozowski, *et al.* 1997). Whether the particular conformation adopted by H12 is directed by ligand as predicted by the structural role of the hormone, through rearrangments of residues within the hydrophobic core, or through direct contacts to H12 which preclude the active conformation, was unfortunately not addressed in the paper. Hopefully, publication of other paired agonist/antagonist structures (Shiau *et al.*, in preparation) will answer that question.

Chapter Two

Structure and Specificity of Nuclear Receptor : Coactivator Interactions



ABSTRACT

Combinatorial regulation of transcription implies the flexible yet precise assembly of multiprotein regulatory complexes in a signal-responsive manner. Biochemical and crystallographic analyses revealed that the thyroid hormone receptor ligand binding domain (LBD), upon hormone binding, forms a hydrophobic groove that interacts with an amphipathic α-helix from GRIP1, a coactivator. The complementary interface on GRIP1 is specified by an LxxLL motif and by residues immediately adjacent to that motif. Parallel studies with the glucocorticoid receptor LBD indicated that these same features are employed differently among different receptor-GRIP1 partners. Such interactions of amphipathic α-helices with hydrophobic grooves also define protein interfaces in other regulatory complexes. We suggest that the use of these common structural elements imparts flexibility to combinatorial regulation, whereas side chains at the interface impart specificity.

INTRODUCTION

Transcriptional regulatory factors integrate input from multiple signals, producing distinct regulatory patterns in different signaling contexts. Regulators typically function in multiprotein regulatory complexes, and the signals evoke structural changes that facilitate or preclude particular protein-protein interactions. By this view, functional regulatory complexes would be structurally dynamic, their composition governed by the specific DNA binding sites arrayed within a given genomic response element, by the particular mixture of regulatory factors produced in a given cell type, and by the physiologic status of that cell as represented by the activities of signaling networks that influence regulatory factor activities (Yamamoto *et al.* 1992). Such a "mixed assembly" model for combinatorial regulation implies that DNA binding regulatory factors, and their various coactivators and corepressors, must interact rather flexibly, to enable assembly into multiple final complexes, yet also quite specifically, to ensure precise assembly into the appropriate complex. How are these conditions satisfied at the molecular level? We have approached this question by studying nuclear receptors, a class of regulatory factors that binds to signals, to response elements and to protein cofactors.

The ligand binding domains (LBD) of nuclear receptors are signal responsive regulatory modules, adopting distinct conformations as aporeceptors, agonist-bound or antagonist-bound species, which support or preclude interactions with proteins such as chaperones, corepressors or coactivators (Moras and Gronemeyer 1998). Within their normal context of intact receptors, LBDs can control virtually all receptor activities, including nuclear localization and DNA binding (Yamamoto *et al.* 1988), and even as isolated domains, LBDs remain functional as compact "molecular switches". Structural analyses of the LBDs from receptors for retinoids (RAR and RXR) (Bourguet, Ruff et al. 1995; Renaud *et al.* 1995), thyroid hormone (TR) (Wagner *et al.* 1995), estrogen (ER)

(Brzozowski *et al.* 1997) , and progesterone (PR) (Williams and Sigler 1998) revealed a common overall fold for the domain, despite substantial sequence divergence (Wurtz *et al.* 1996). That ligand binding induces conformational changes, affecting especially the position of an α-helix at or near the LBD C-terminus, can be inferred from comparisons of structures of the unliganded RXR with liganded RAR and TR LBDs, and of the agonist- and antagonist-bound ER LBD. This helix, usually denoted helix 12, is an essential component of a ligand-dependent transcriptional activation function, AF-2, within the LBD (Danielian, White et al. 1992; Barettino, Vivanco Ruiz et al. 1994; Durand, Saunders et al. 1994; Tone, Collingwood et al. 1994).

Agonist-bound LBDs can bind coactivators from the p160 family, which includes at least three distinct members, SRC-1 (Onate *et al.* 1995) (NcoA-1) (Kamei *et al.* 1996), p/CIP (Torchia *et al.* 1997) (AIB1 (Anzick *et al.* 1997), TRAM-1 (Takeshita *et al.* 1997), RAC3 (Li *et al.* 1997), ACTR (Chen *et al.* 1997)), and TIF2 (Voegel *et al.* 1996) (GRIP1 (Hong *et al.* 1996; Hong *et al.* 1997), NcoA-2 (Torchia, Rose et al. 1997)) (Glass *et al.* 1997). These ~160kD proteins include N-terminal bHLH and PAS domains, domains for the interaction with nuclear receptors (NID) and CBP, and C-terminal activation domains (Figure 2.1*a*) (Ding, Anderson et al. 1998; Kalkhoven *et al.* 1998; Voegel, Heine et al. 1998).

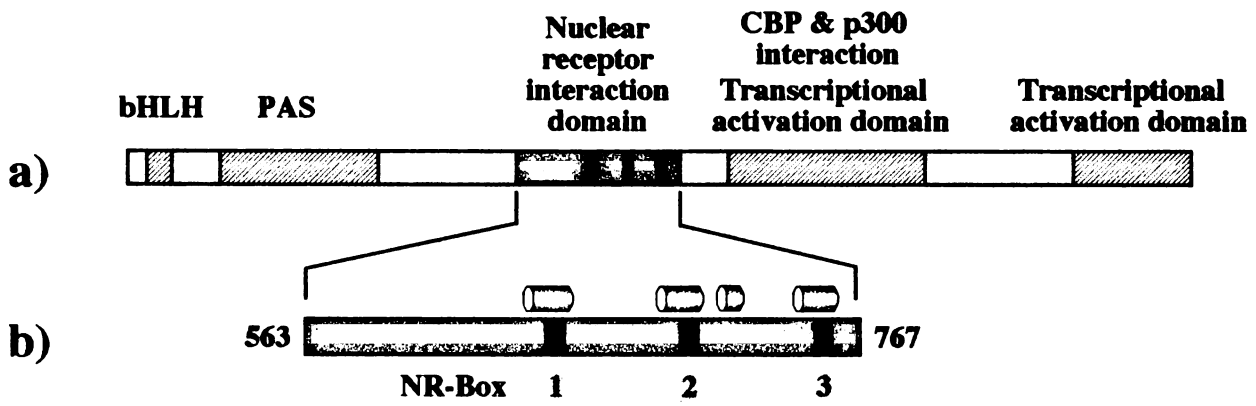
Figure 2.1 p160 family of coactivators

(a) Functional domains of p160 family coactivators. (Ding *et al.* 1998; Voegel *et al.* 1998).

(b) The nuclear receptor interaction domain (NID) of GRIP1. The NID of GRIP1 (563-767) contains four predicted α-helices (according to PhD)(Rost *et al.* 1994); three of them include the conserved LxxLL motifs of NR-boxes 1, 2 and 3.

(c) Sequence alignment of LxxLL motifs in members of the p160 coactivator family. Motif leucine residues are green, box-specific conserved residues are red. GRIP1 (Hong, Kohli et al. 1996; Hong, Kohli et al. 1997), pCIP (Torchia, Rose et al. 1997) and SRC-1 (Onate, Tsai et al. 1995) were taken as representatives for the three distinct classes of coactivators in the p160 family. Predicted α-helices are shown for each box.

Figure 2-1 p160 family of coactivators



c)

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1   Grip1       AEGHSRLHDSKGQTKLLQLLTTKSEQMEPSPLAS
    p/CIP       SETPRGPLESKGHKKLLQLLTCSSEDRGHSSLTN
    SRC1        SEGDSKY--SQTSHKLLQLLTTTAEQQLRHADID

    Consensus   .-........S....KLLQLLT...-.........

2   Grip1       PGSTHGTSLKEKHKILHRLLQDSSSPVDLAKLTA
    p/CIP       TSNVHGSLLQEKHRILHKLLQNGNSPAEVAKITA
    SRC1        TCPSSHSSLTERHKILHRLLQE.GSPSDITTLSV

    Consensus   ........L.E+H+ILH+LLQ...SP.-h..h..

3   Grip1       EPASPKKKE---NALLRYLLDKDDTKDIGLPEIT
    p/CIP       EQLSPKKKE--NNALLRYLLDKDDPSDALSKELQ
    SRC1        ELDAAKKKESKDHQLLRYLLDKDEKDLRSTPNLC

    Consensus   E....KKKE.....LLRYLLD+D-........h.
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In p160 coactivators, and in unrelated coactivators such as TIF1 or RIP140, interaction with nuclear receptors is mediated by multiple LxxLL motifs, each residing within distinct patches of conserved sequence termed NR boxes (Figure 2.1*b, c*) (Le Douarin *et al.* 1996; Heery *et al.* 1997; Torchia, Rose et al. 1997; Ding, Anderson et al. 1998; Voegel, Heine et al. 1998). Particular nuclear receptors show overlapping but distinct preferences for individual NR-boxes (Le Douarin, Nielsen et al. 1996; Torchia, Rose et al. 1997) .

We sought to define the structural and molecular basis for the interaction of nuclear receptors with the LxxLL motif of p160 coactivators, and to identify the determinants of specificity of these interactions. To this end we studied the interaction of the p160 coactivator GRIP1 with TR or with the glucocorticoid receptor (GR) *in vitro*, and solved the structure of the TRβ LBD in complex with a peptide containing NR-box 2 of GRIP1.

RESULTS

GRIP1 interaction with TRβ

We purified a 205 aa fragment of GRIP1 (aa 563-767), denoted NID (nuclear receptor interaction domain), that was readily soluble and contained three NR-boxes (NR-box 1-3) (Figure 2.1*b*). A secondary structure prediction program (Rost, Sander et al. 1994) placed each NR-box within separate α-helical regions, in each case close to the C-terminus of the helix.

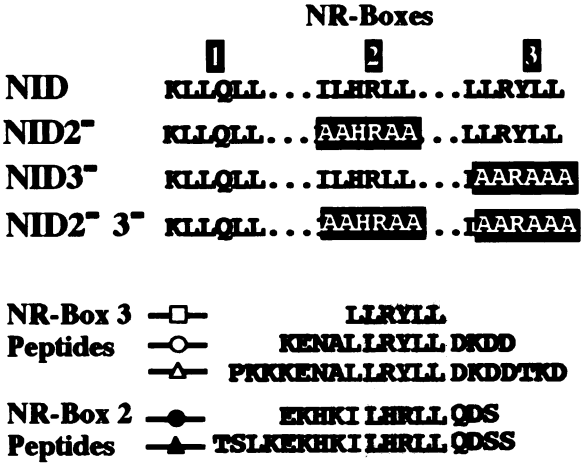
To measure the interaction between TRβ and GRIP1 *in vitro*, fusions bearing glutathione S-transferase (GST) and NID or NID derivatives bearing mutant NR-boxes were constructed, expressed, purified and used to monitor interactions with ^{35}S-labeled TRβ or TRβ LBD by retention on glutathione agarose; full length TRβ or TRβ LBD bound the NID of GRIP1 with equal affinity and specificity (data not shown). Purification of the GST-NID fusions to near homogeneity enabled quantitative comparisons of interactions between different GRIP1 derivatives and receptors; each assay was performed with known concentrations of the GST-NID and receptor derivatives.

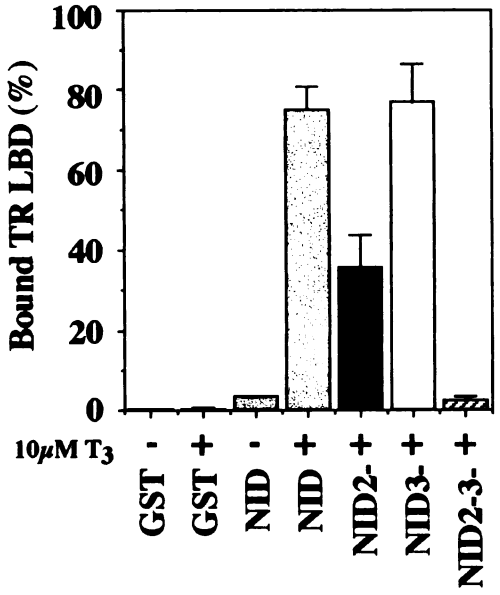
The TRβ LBD:NID interaction was strongly hormone-dependent (Figure 2.2) .

Figure 2.2 GRIP1 NR-boxes 1, 2 and 3 interact differentially with TRβ LBD

GST denotes an isolated GST domain. NID is the GST fusion of the GRIP1 fragment (563-767)His_6 that contains NR-boxes 1, 2 and 3 (gray). In NID2$^-$ (black) or NID3$^-$ (white) the bulky hydrophobic residues of NR-box 2 (ILHRLL) or NR-box 3 (LLRYLL) were replaced by alanine yielding (AAHRAA) and (AARAAA), respectively. NID2$^-$3$^-$ (diagonally striped) contains replacement of both NR-boxes 2 and 3. Assays were carried out using 10 nM TRβ LBD, synthesized and labeled with ^{35}S methionine *in vitro* and 1.6 μM purified, glutathione-agarose bound GST-NID proteins either in the absence (–) or presence (+) of 10 μM T_3. The yield of bound receptor is given as percentage of the input.

Figure 2-2 GRIP1 NR-boxes 1, 2 and 3 interact differentially with TRβ LBD





Substitution of the bulky hydrophobic residues of NR-box 3 by alanine (NID3$^-$), did not reduce binding of TRβ LBD significantly, whereas similar substitutions in NR-box 2 (NID2$^-$) resulted in a 50% loss of TRβ LBD binding. In the absence of functional NR-boxes 2 and 3 (NID2$^-$3$^-$), no ligand dependent binding to TRβ LBD was detected under our experimental conditions, suggesting that multiple binding sites are required or that NR-box 1 is not a binding site for TRβ LBD. The latter possibility was strongly supported by subsequent peptide competition assays (see below). The observed preference of TRβ LBD for NR-box 2 is consistent with previous results from yeast two-hybrid and mammalian transfection assays (Ding, Anderson et al. 1998). Thus, our *in vitro* assay reflects the selectivity of TR:GRIP1 interactions observed *in vivo*.

Peptide competition of NR-box TRβ LBD interaction

We compared the ability of peptides containing individual NR-boxes with different lengths of adjacent sequences to compete the interaction of NID3$^-$ with TRβ LBD. The central hexapeptide of NR-box 3, LLRYLL, competed the interaction of NID3$^-$ with TRβ LBD with very low efficiency, demonstrating that an isolated motif is not sufficient for binding to the receptor (Figure 2-3). Extending this hexapeptide to the 14-mer KENALLRYLLDKDD decreased the IC_{50} for competition by more than 60-fold, from

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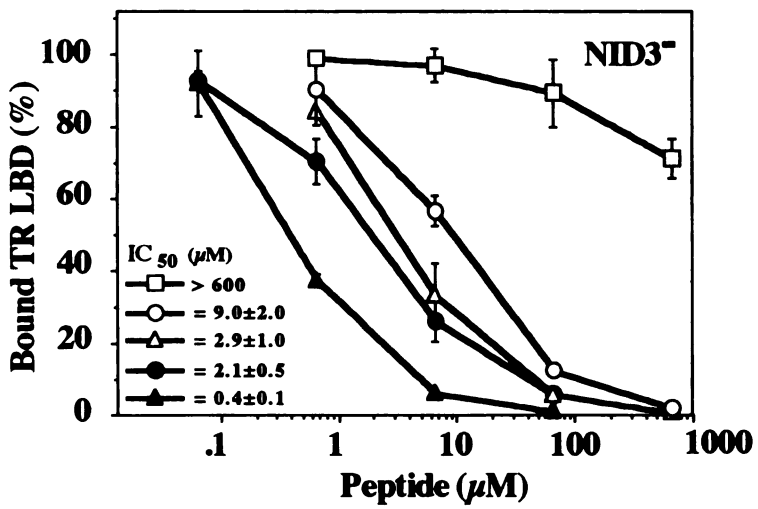
Figure 2-3 Peptides containing NR-boxes compete the TRβ LBD : NID interaction

In vitro translated, labeled TRβ LBD (10 nM) was incubated with 1.6 μM glutathione agarose-bound GST-NID3$^-$ in the presence of 10 μM T_3 and increasing concentrations of NR-box 2 peptides EKHKILHRLLQDS (solid circle) or TSLKEKHKILHRLLQDSS (solid triangle), or NR-box 3 peptides LLRYLL (open square), KENALLRYLLDKDD (open circle) or PKKKENALLRYLLDKDDTKD (open triangle). The conserved, hydrophobic interaction motif is highlighted. Bound receptor is shown relative to the amount retained in the absence of peptide. The data and calculated IC_{50} values represent the average and standard deviation of three independent experiments.

Figure 2-3 Peptides containing NR-boxes compete the TRβ LBD : NID interaction



>600 μM to 9.0±2.0 μM; further extension of the peptide to PKKKENALLRYLLDKDDTKD decreased the IC_{50} only 3-fold further, to 2.9±1.0 μM. Similarly, the two NR-box 2 peptides EKHKILHRLLQDS or TSLKEKHKILHRLLQDSS were potent competitors of the TRβ LBD:NID3$^-$ interaction (IC_{50} = 2.1±0.5 μM or 0.4±0.1 μM, respectively), demonstrating that 13 and 14 amino acid peptides encompassing NR-boxes 2 and 3, respectively, are sufficient to interact with TRβ LBD. Competition of the TRβ LBD:NID interaction by the NR-box 2 peptide EKHKILHRLLQDS and NR-box 3 peptide KENALLRYLLDKDD yielded similar results (IC_{50} = 2.2±0.7 μM or IC_{50} = 6.0±2.7 μM, respectively) (data not shown).

Affinity and specificity of the NID for TRβ LBD

Consistent with the observation that NID3$^-$ interacts more efficiently with TRβ LBD than does NID2$^-$, peptides containing NR-box 2 were more potent inhibitors of the TRβ LBD:NID3$^-$ interaction than peptides containing NR-box 3 (Figure 2.3). To quantify these differences, we titrated the levels of NID3$^-$ and NID2$^-$, and determined that the TRβ LBD binds 3- to 4-fold stronger to NR-box 2 (EC_{50} = 0.9±0.2 μM) than NR-box 3 (EC_{50} = 3.2±0.9 μM) (Figure 2.4) . Based on the Cheng and Prussoff equation , the apparent affinities of TRβ LBD for the NR-box 2 peptide EKHKILHRLLQDS ($k_D$app = 0.8±0.3 μM) and for NR-box 3 peptide KENALLRYLLDKDD ($k_D$app = 3.2±1.2 μM) were indistinguishable from affinities for the full domain derivatives NID3$^-$ or NID2$^-$,

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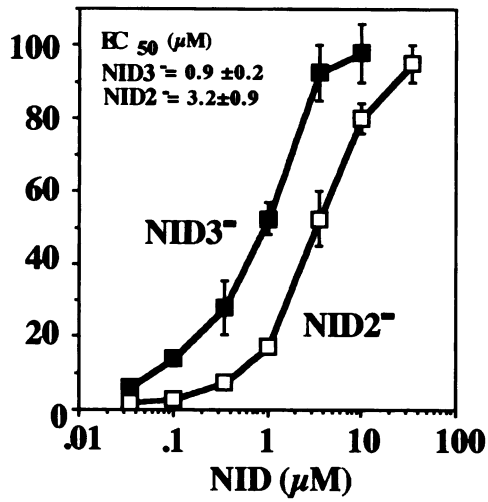
Figure 2-4 NR-box 2 interacts with TRβ LBD with higher affinity than NR-box 3

In vitro translated, labeled TRβ LBD (10 nM) was incubated in the presence of 10 μM T_3 and various concentrations of purified and glutathione agarose bound GST-NID2$^-$ (open square) or GST-NID3$^-$ (solid square) lacking either a functional NR-box 2 or 3, respectively. As in Figure 2.2, the amount of bound receptor is relative to the receptor input. The data represent the average and range of two independent experiments.

Figure 2-4 NR-box 2 interacts with TRβ LBD with higher affinity than NR-box 3



respectively. As expected, a peptide containing NR-box 1, which in the context of the NID did not interact with TRβ LBD, competed the interaction of GRIP1 with TRβ LBD only at very high concentrations (EC_{50} = 130μM) (data not shown). We conclude that the interaction of these NR-boxes with TRβ LBD is highly localized to the LxxLL motif and sequences immediately adjacent, and that the preference of TRβ LBD for particular NR-boxes depends on the sequence rather than the structural context of the NR-boxes within the NID.

Structure of the TRβ LBD:NR-box 2 peptide complex

Having demonstrated that peptides containing NR-boxes mimic the interaction of the TRβ LBD with the GRIP1 NID domain, we cocrystallized the 13 amino acid NR-box 2 peptide KHKILHRLLQDSS with TRβ LBD, under conditions where the peptide or TRβ LBD alone precipitated. The structure of the TRβ LBD:NR-box 2 peptide complex was determined by molecular replacement using the structure of the human TRβ LBD (Wagner, unpublished), and refined to an R-factor of 25% (Table 1).

Table 2-1 Data collection, phasing, and refinement statistics for the human β TR LBD : NR box 2 peptide complex

Data collection

Data set	Resolution (Å)	Reflections		Coverage (%)	*R_{sym}
		measured	unique		
Native	3.6	35565	8490	96.3	0.077

Rotation search

Search model		Euler angles (°)			†Correlation coefficient	
		Θ_1	Θ_2	Θ_3	Highest peak	Highest false peak
TRβ LBD	M1	60.12	80.68	241.90	16.3	-
	M2	9.93	87.70	180.6	15.9	14.2

Translation search

	Fractional coordinates			‡Translation function	
	x	y	z	Highest peak (σ)	Highest false peak (σ)
M1	0.522	0.428	0.250	19.52	10.02
M2	0.200	0.932	0.119	26.11	5.77

	Resolution	Reflections	§R	§R_{free}
F > 2σ	25 - 3.7Å	7614	24.9	29.9

R.M.S deviations bonds 0.0098 Å angles 1.60°

*$R_{sym} = \Sigma_h \Sigma_i | I_{h,i} - \langle I_h \rangle | / \Sigma I_h$ for the intensity (I) of i observations of reflection h.

†Correlation coefficient = $\Sigma_h E_o^2 E_c^2 - \langle E_o^2 \rangle \langle E_c^2 \rangle / \{\Sigma_h (E_o^2 - \langle E_o^2 \rangle)^2 \Sigma_h (E_c^2 - \langle E_c^2 \rangle)^2\}^{1/2}$

‡Translation function $(t_a,t_b,...) = \Sigma_h (|E_o(h)|^2 - \Sigma_h \langle |E_c(h)|^2 \rangle) (|E_c (h,t_a,t_b,...)|^2 - \langle |E_c(h)|^2 \rangle)$

where E_o represents the normalized observed structure factor amplitudes, and E_c represents the normalized structure factors for the search model in a triclinic unit cell with dimensions identical to that of the crystal. The reported peak height represents the value of the function for the translations (t_a,t_b) of the NCS monomers, divided by the rms value of the translation function density.

§R factor = $\Sigma | F_{obs} - F_{calc} | / \Sigma | F_{obs} |$. R_{free} is calculated the same as the R factor, using 10% of the reflections that were set aside for cross validation and not used in refinement.

The asymmetric unit of the crystal contained two monomers of the TRβ LBD and two NR-box 2 peptides (Figure 2.5*a,b)*[r].The structure of the TRβ LBD consisted of 12 α-helices and 4 β-strands organized in three layers, as observed for the rTRα LBD (Wagner, Apriletti et al. 1995). Residues K688-Q694 of the NR-box 2 peptide formed an amphipathic α-helix of nearly 3 turns, with residues H687 and D695-S698 on either end in extended coil conformation (peptide A) or disordered (peptide B). While the resolution of the data precluded assignment of main-chain hydrogen bonds, it was clear from the periodicity of the side chain density that the central residues form an α-helix (Figure 2.6) . In the absence of the TRβ LBD, the far UV-CD spectrum of the NR-box 2 peptide indicated a random coil conformation (data not shown); thus helix formation appears to be induced by complex formation.

Figure 2-5 Contents of the asymmetric unit and detail

(a) The contents of the asymmetric unit of the crystallized TRβ LBD:NR-box 2 complex. The crystal lattice consists of a repeating unit containing a 2:2 complex of TRβ LBD and NR-box 2 peptide. The two monomers of the TRβ LBD are shown as a ribbon drawing, with monomer A in light gray, and monomer B in dark gray. The positions of the two NR-box 2 peptides are outlined, and the peptides depicted as a magenta secondary structure ribbon. The interaction of peptide A with monomer A, and of peptide B with monomer B, observe the same non-crystallographic symmetry relation as the two TRβ LBD monomers. The ligand, T_3, is shown in a space-filling representation.

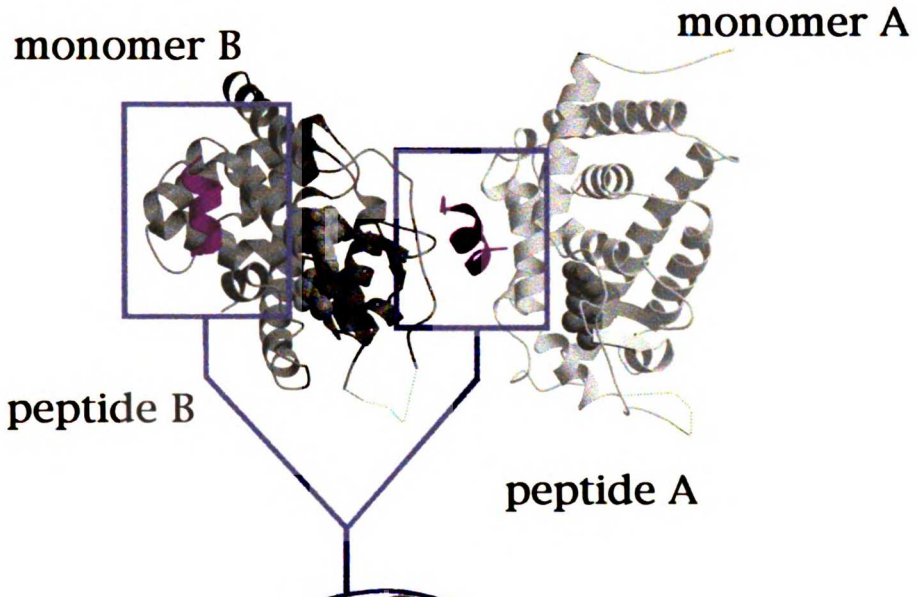
(b) Detail of the interaction of the NR-box 2 peptide A with the monomer A of the TRβ LBD. The NR-box 2 peptide binds the TRβ LBD in a hydrophobic groove defined by helices H3, H4, H5, and H12. Non-interacting portions of the TRβ LBD, and of neighboring monomer B, are omitted for clarity. The side chains of the hydrophobic residues I689, L690, L693, L694 of the NR-box 2 peptide are drawn to emphasize the hydrophobic interactions observed in both bound peptides.

Figure 2-6 Electron density for NR-box 2 peptide A

Initial Fo-Fc electron density map for the NR-box 2 peptide A, using phases calculated from the TRb LBD model following 1 cycle of positional refinement against a maximum likelihood target in CNS (A.T.Brunger, personal communication), with strict non-crystallographic symmetry enforced. The electron density is contoured at 3.5σ (blue) and 2.5σ (cyan). The peptide appears as a stick representation, using the final refined atomic model.

Figure 2-5 Contents of the asymmetric unit and detail

a



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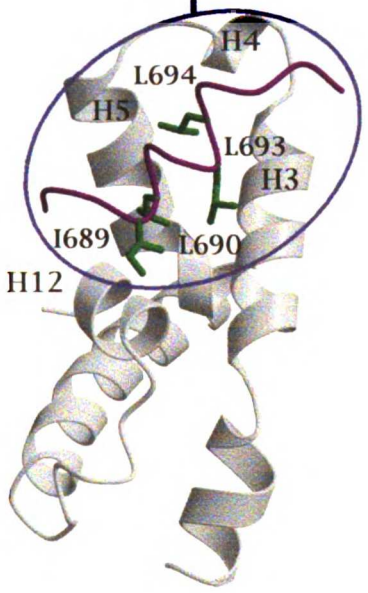
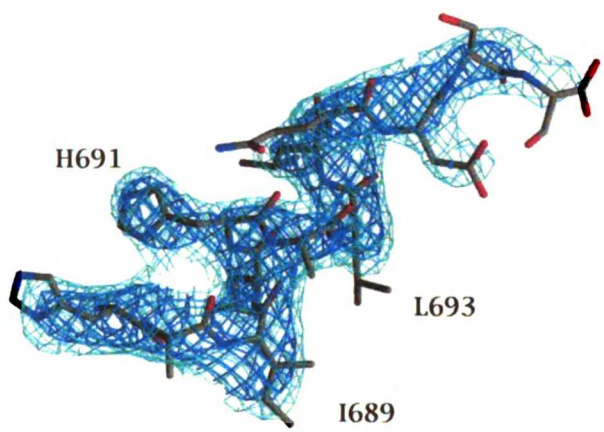


Figure 2-6 Electron density for NR-box 2 peptide A



One of the NR-box 2 peptides, peptide A, bridged the two TR monomers: the hydrophobic face of the α-helix contacted TRβ LBD monomer A at H3, H4, H5, and H12, whereas the hydrophilic face of peptide A contacted monomer B at the hairpin turn preceding ß-strand S3. In contrast, the interaction of peptide B with TRβ LBD was restricted to monomer B, also contacting H3, H4, H5, and H12. The interface common to both TRβ LBD:NR-box 2 peptide complexes buried the LxxLL motif (GRIP1 residues L690, L693, and L694) against a hydrophobic surface of the receptor LBD (Figure 2.5*b*). The interactions displayed by both peptides in the asymmetric unit are likely specific for the interaction of the peptide with the TRβ LBD, and not driven by crystallization. This conclusion is supported by mutational studies that identified residues in the LBDs of various nuclear receptors that are important for ligand-dependent transcriptional activation and coactivator binding (Tone, Collingwood et al. 1994; Renaud, Rochel et al. 1995; Collingwood *et al.* 1997; Henttu *et al.* 1997; Saatcioglu *et al.* 1997; Feng *et al.* 1998): all map to the common interface of the TRβ LBD with the NR-box 2 peptides A and B. In contrast, the interaction of the hydrophilic face of peptide A with monomer B likely represents a gratuitous crystal contact.

The TRβ LBD:LxxLL interface

The surface of TRβ LBD that interacted with the NR-box 2 peptide was formed by 16 residues from four helices, H3, H4, H5, and H12, which packed against one another to create a shallow, hydrophobic groove (Figure 2.7). The residues that formed the groove

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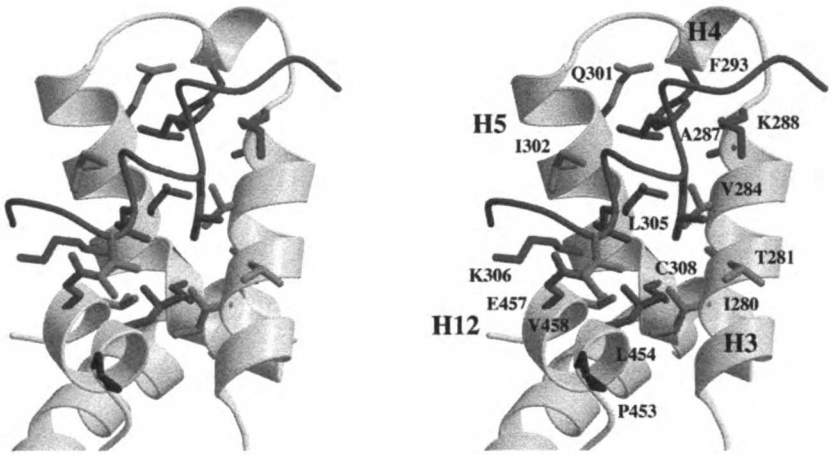
Figure 2-7 The TRβ LBD : NR-box 2 peptide interface

The side chains of those residues of the TRβ LBD within 4.5Å of the NR-box 2 peptide are labeled. Colors reflect chemical properties of the side chains: acidic residues are red, basic residue are blue, aliphatic residues are green, aromatic residues are brown, and polar residues are orange. The peptide is depicted as a Cα trace, with the side chains of ILxxLL motif shown explicitly. The view is identical to that of Figure 2.5.

Figure 2-7 The TRβ LBD : NR-box 2 peptide interface



are: I280, T281, V283 , V284, A287, and K288 from H3; F293 from H4; Q301, I302, L305, K306 and C308 from H5; and L454, E457, V458, and F459 from H12 (Figure 2.7). Hydrophobic residues formed the floor of the groove, with charged residues lining the rim (Figure 2.8 *a,b*) .

NR-box 2 peptide A bound at the junction of H3 and H12 of LBD monomer A (Figure 2.7). L690 was inserted into a shallow depression at the base of the groove, making van der Waals contact with L454 and V458 of H12, while I689 packed against L454 of H12 outside the edge of the groove; L454 interdigitated between the two peptide residues. One further turn C-terminal along the peptide A α–helix, L693 and L694 packed into complementary pockets within the groove. L693 formed a van der Waals contact with V284 of H3, while L694, bound more deeply in the groove, contacted F293 and L305 of H4 and H5 (Figure 2.7). The TRβ LBD:NR-box 2 interface buried 980 $Å^2$ of surface area, most of which is hydrophobic. The surface generated by the three conserved leucines (L690, L693, L694) of the NR-box 2 peptide was highly complementary to the corresponding surface in the TRβ LBD (Figure 2.9). As noted above, similar hydrophobic interactions were observed between peptide B and monomer B.

Figure 2-8 Molecular and electrostatic surface of the TRβ LBD

(a) The side chains of the leucines residues from the NR-box 2 peptide fit within a hydrophobic groove on the surface of the TRβ LBD formed from helices H3, H4, H5, and H12, whereas the side chain of the non-conserved isoleucine residue packs against the outside edge of the groove. The remainder of the peptide is shown as main chain.

(b) Areas of positive electrostatic potential are shown in blue; areas of negative electrostatic potential are shown in red. Individual charged residues of the TRβ LBD at the NR-box interface are labeled. The NR-box 2 peptide is shown as in Figure 2.7. Electrostatic and surface calculations used GRASP (Nicholls, Sharp et al. 1991).

Figure 2-9 Complementarity of the TRβ LBD:LxxLL interface

The side chains of the NR-box 2 ILxxLL motif are shown in a CPK representation, with the main chain of the peptide drawn as a Cα worm. The three leucine residues fit into pockets on the molecular surface of the TRβ LBD, depicted as mesh, while the nonconserved isoleucine residue rests on the edge of the surface cleft.

Figure 2-8 Molecular & electrostatic surface of the TRβ LBD

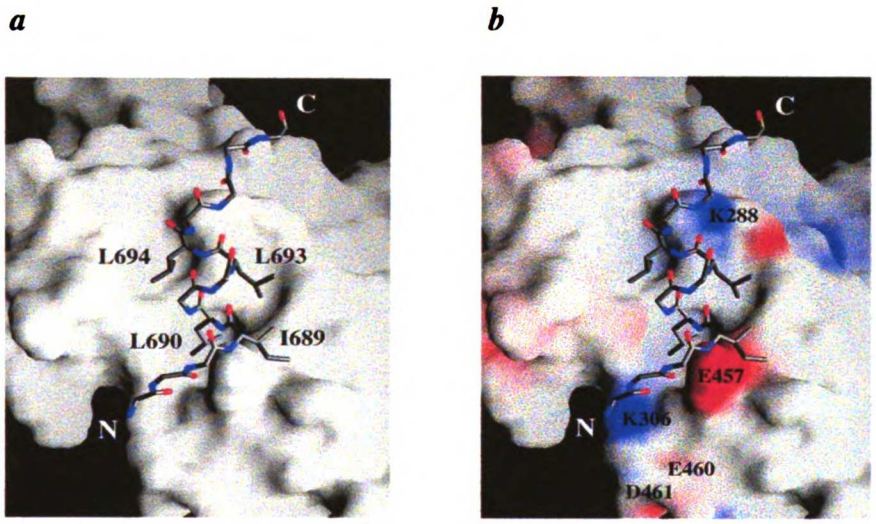
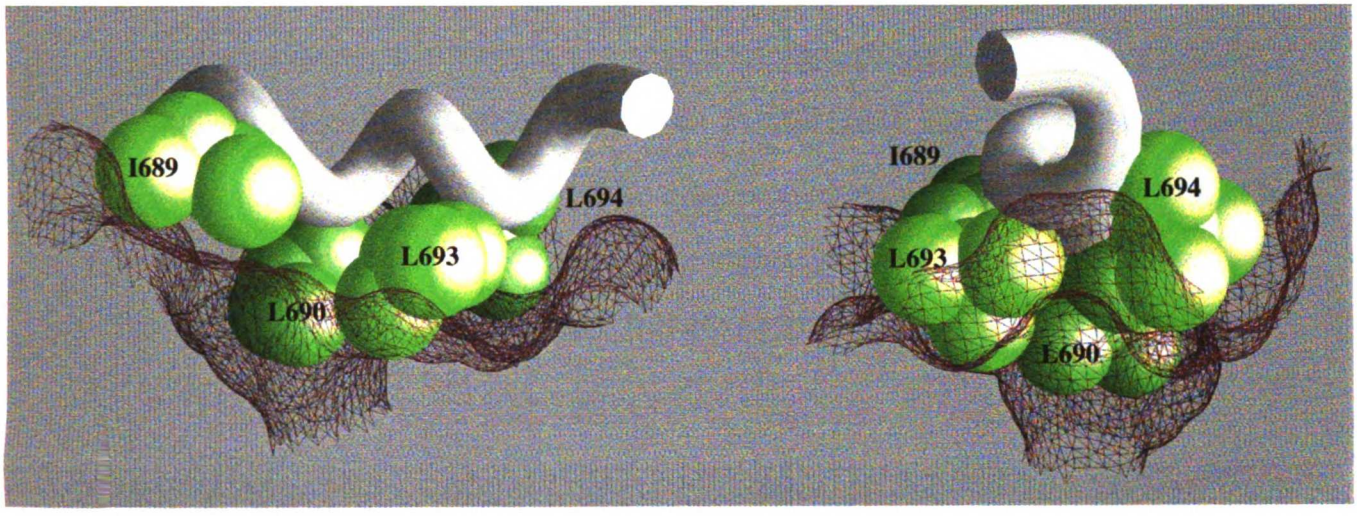


Figure 2-9 Complementarity of the TRβ LBD:LxxLL interface



The crucial role of the hydrophobic residues within the LxxLL motif for the formation of a complex with the LBD of nuclear receptors is supported by several mutational studies *in vitro* and *in vivo*, showing that substitution of multiple leucine residues of the motif by alanines abrogates physical and functional interactions between p160 family coactivators and nuclear receptors (Le Douarin, Nielsen et al. 1996; Heery, Kalkhoven et al. 1997; Torchia, Rose et al. 1997). To investigate the role of the hydrophobic residues in NR-box 2, we replaced individual residues of the ILHRLL motif by alanine in the background of NID3$^-$. Replacement of any one of the three leucines severely compromised binding to TRβ LBD (Figure 2.10, whereas replacement of the isoleucine specific to NR-box 2, which is not as deeply buried as the conserved leucine residues, reduced binding to a lesser degree. Parallel results were obtained by competing the interaction of TRβ LBD with NID3$^-$ using peptides in which either IL, HR or LL of the NR-box 2 motif were replaced pairwise by alanines (Figure 2.11). Whereas the peptides containing the IL or LL replacements failed to compete for TRβ LBD even at very high

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Figure 2-10 Individual leucine residues of the LxxLL motif are crucial for binding of GRIP1 NID to TRβ LBD

1.6 μM (solid bars) or 4.0 μM (hatched bars) of glutathione agarose-bound GST-NID3$^-$ or variants containing alanine substitutions of individual hydrophobic residues of the NR-box 2 ILxxLL motif (ALxxLL, I689A; IAxxLL, L690A; ILxxAL, L693A; ILxxLA, L694A; IAxxLA, L690A+L694A), were incubated with *in vitro* translated, labeled TRβ LBD in the presence of 10 μM T_3; mutations are in boldface. The amount of bound receptor is relative to the receptor input. The figure presents the results of a representative experiment.

Figure 2-11 Pairwise or single conservative substitutions of the ILxxLL leucine residues drastically reduce the affinity of NR-box 2 peptides for TRβ LBD

Interaction of 1.6 μM glutathione agarose-bound NID3$^-$ with 10 nM *in vitro* translated, labeled TRβ LBD + 10 μM T_3 was competed with increasing concentrations of variants of the NR-box 2 peptide KHKILHRLLQDSS, containing either (b) pairwise alanine substitutions KHKAAHRLLQDSS, KHKILAALLQDSS, KHKILHRAAQDSS, or (c) single phenylalanine substitutions of conserved residues of the hLxxLL motif (KHKFLHRLLQDSS, KHKIFHRLLQDSS, KHKILHRFLQDSS, KHKILHRLFQDSS); mutations are in boldface. The amount of bound receptor is relative to the amount of retained receptor in the absence of peptide. The data represent the average and standard deviation of three independent experiments.

Figure 2-10 Individual leucine residues of the LxxLL motif are crucial for binding of GRIP1 NID to TRβ LBD

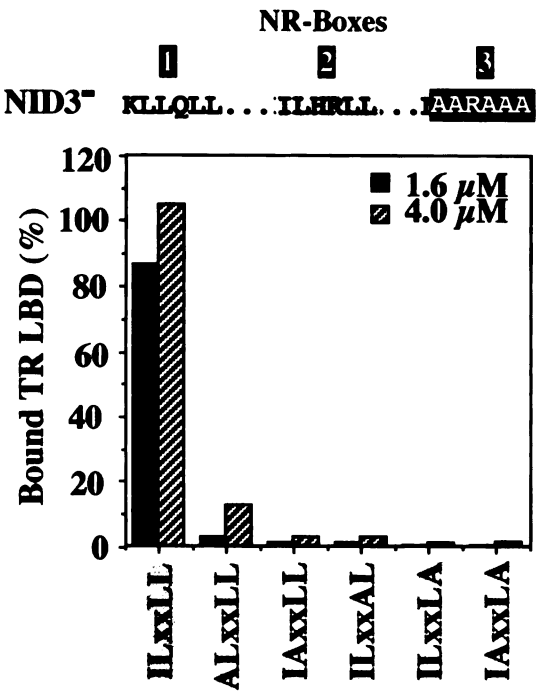
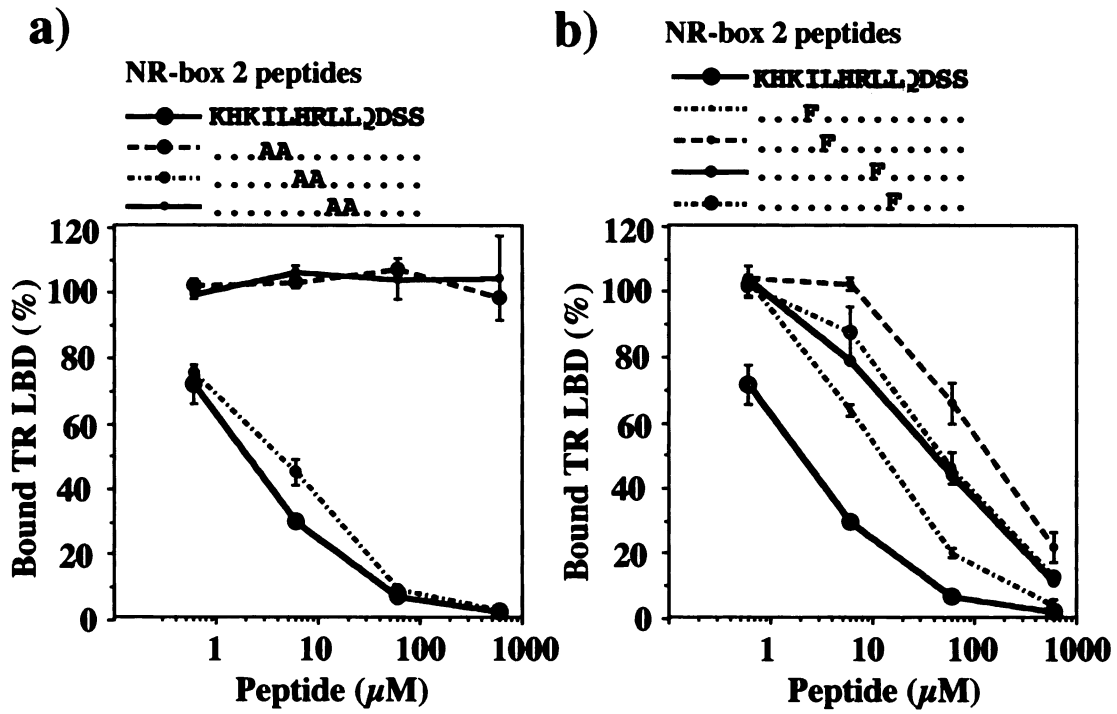


Figure 2-11 Pairwise or single conservative substitutions of the ILxxLL leucine residues drastically reduce the affinity of NR-box 2 peptides for TRβ LBD



concentrations, replacement of the "HR spacer" by alanines had little effect on competition, consistent with the α-helical structure of the motif. In the TRβ:GRIP1 complex, the NR-box 2 residues I689 and L690 packed against L454 and V458 of H12 whereas one helical turn further in the motif, L693 and L694 contacted V284, F293 and L305 from H3, H4 and H5, respectively, of the TRβ LBD (Figure 2.7). The strong dependence of the interaction on the integrity of the entire motif suggests that these interactions are not independent and drive the interaction through cooperative rather than additive contributions.

Replacement of single leucine residues of NR-box 2 by phenylalanine reduced competition by the mutant peptides for TRβ LBD by 60-100-fold relative to wild type peptide, whereas replacement of the isoleucine resulted in about a 10-fold reduction (Figure 2.11). Therefore, efficient interaction of TRβ with GRIP1 relies not simply on the hydrophobicity of the LxxLL motif, but rather on stereochemical properties of the leucine side chains, consistent with the observed structural complementarity of the interacting surfaces (Figure 2.9). The lesser effect on affinity by the replacement of I689 by phenylalanine may be explained by the location of I689 on the rim of the hydrophobic groove, where steric changes may be more easily accommodated. The stronger effect of the replacement of L690 correlates well with position of this residue deep within the hydrophobic groove. It is notable, however, that mutant peptides bearing bulky hydrophobic motifs other than LxxLL retain some affinity, albeit substantially weaker, for the TRβ LBD.

The role of the adjacent sequences

Our analyses demonstrated that the conserved LxxLL motif of NR-box 2 is necessary for strong interaction of GRIP1 with TRβ LBD. However, the lower affinity of the NR-box 3 hexapeptide LLRYLL for TRβ LBD (Figure 2.3) implied that an isolated

LxxLL motif is not sufficient. Sequence identity among all the NR-boxes of the p160 coactivator family is limited to the conserved leucine residues of the LxxLL motif, but for each NR-box, the sequence conservation extends into adjacent residues (Figure 2.1*c*).

NR-box 2 interacted with the TRβ LBD with a 4-fold higher affinity than NR-box 3. To determine whether this affinity differential reflects differences in the respective hydrophobic motifs (NR-box 2: ILHRLL, NR-box 3: LLRYLL) or their adjacent sequences, we constructed a chimeric peptide containing the NR-box 3 motif in the context of adjacent sequences from NR-box 2. This peptide competed the interaction of NID3^{-} with TRβ LBD with the same potency as the intact NR-box 2 peptide, demonstrating that the higher affinity of TRβ LBD for GRIP1 NR-box 2 is determined by the sequences adjacent of the LxxLL motif (Figure 2.12) .

In principle, adjacent sequences might also decrease the affinity of a motif-driven interaction. We uncovered an example of such an effect when we constructed a chimeric peptide bearing the NR-box 2 LxxLL motif flanked by sequences normally adjacent to a VP16 Fxxhh motif, which interacts with $TAF_{II}31$ in a complex (Uesugi *et al.* 1997) that is structurally similar to the TRβ LBD:NR box complex. The intact VP16 peptide EFEQMFTDALGIDE did not interact with TRβ LBD even at very high concentrations (Figure 2.12), demonstrating that the hydrophobicity of peptides is not sufficient to mediate

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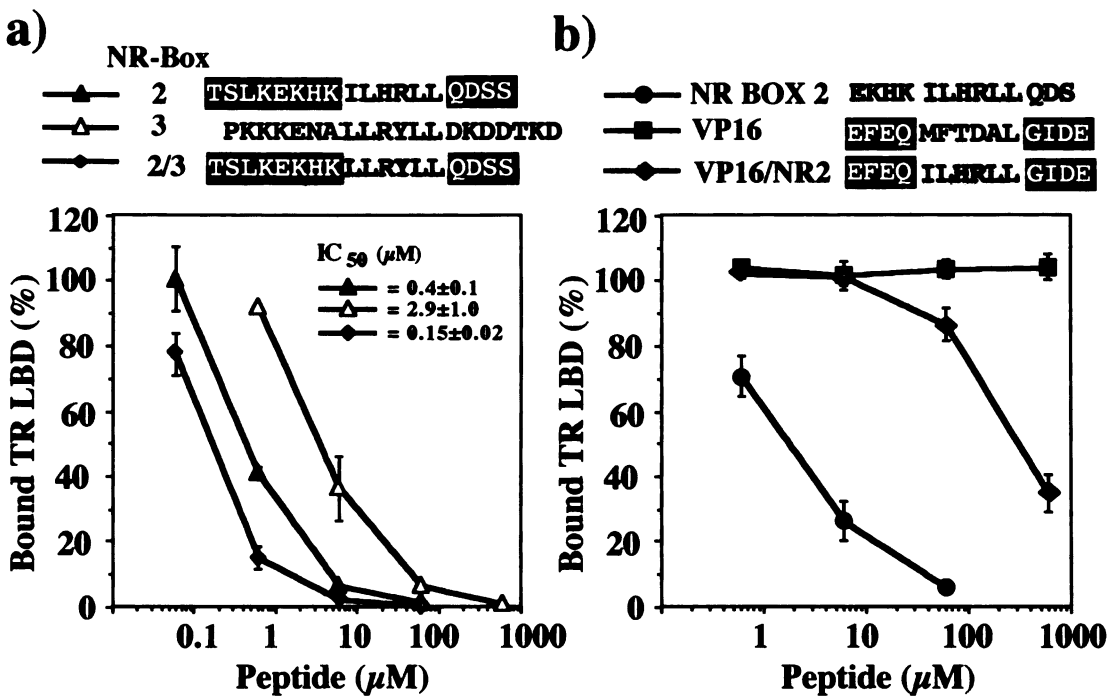
Figure 2-12 Sequences adjacent to the LxxLL motif affect the affinity of the TRβ LBD NR-box 2 interaction

In vitro translated, labeled TRβ LBD (10 nM) + 10 μM T_3 was incubated with 1.6 μM glutathione agarose bound GST-NID3^{-} in the presence of increasing concentrations of peptides containing (a) NR-box 2 TSLKEKHKILHRLLQDSS (solid triangle), NR-box 3 PKKKENALLRYLLDKDDTKD (open triangle), or the LLRYLL motif of NR-box 3 in the context of flanking sequences from NR-box 2 TSLKEKHKLLRYLLQDSS (gray diamond) or (b) NR-box 2 EKHKILHRLLQDS (solid circle), VP16 EFEQMFTDALGIDE (solid square), or the ILHRLL motif of NR-box 2 in the context of the adjacent sequences from the VP16 peptide EFEQILHRLLGIDE (gray diamond). The amount of bound receptor is relative to the amount of retained receptor in the absence of peptide. The data and IC_{50} values represent the average and standard deviation of three independent experiments.

Figure 2-12 Sequences adjacent to the LxxLL motif affect the affinity of the TRβ LBD:NR-box 2 interaction



interaction with TRβ. The chimeric peptide EFEQILHRLLGIDE competed the interaction of TRβ LBD with NID but with an about 100-fold lower potency than the NR-box 2 peptide EKHKILHRLLQDS. We conclude that the affinity of the interaction between the NR-boxes and TRβ LBD depends on both the hydrophobic motif and the adjacent sequences. As single substitutions of the conserved leucine residues of the LxxLL motif by alanines compromise interaction of the GRIP1 NID with TRβ LBD, the contributions of the adjacent sequences appear to depend on the interaction of the hydrophobic motif with TRβ LBD, and are not sufficient to mediate interaction by themselves.

The discrimination of sequences flanking the LxxLL motif by the TRβ LBD likely reflects the interaction of specific NR-box residues with the TRβ LBD interaction surface. In the TRβ LBD:peptide A complex, the positively charged N-terminus of the NR-box 2 peptide KHKILHRLLQDSS followed a shallow groove between H5 and H12 (Figure 2.8). Interaction of the negatively-charged residues at the C-terminus of the TRβ LBD (E460, D461) with the positively charged N-terminus of the NR-box 2 peptide may provide a favourable contact, which would be missing in the case of the NR-box 3 peptide ENALLRYLLDKDD, or repulsive in the case of the NR-box 2/VP16 chimeric peptide EFEQILHRLLGIDE.

To test this hypothesis, we analyzed the interaction of peptides containing NR-box 2 or 3 with a mutant TRβ LBD, in which the residues E460 and D461 were substituted by threonine and asparagine, respectively, which are the homologous residues in GR (Figure 2.13) . Indeed, the affinity for the NR-box 2 peptide of this mutant was decreased about

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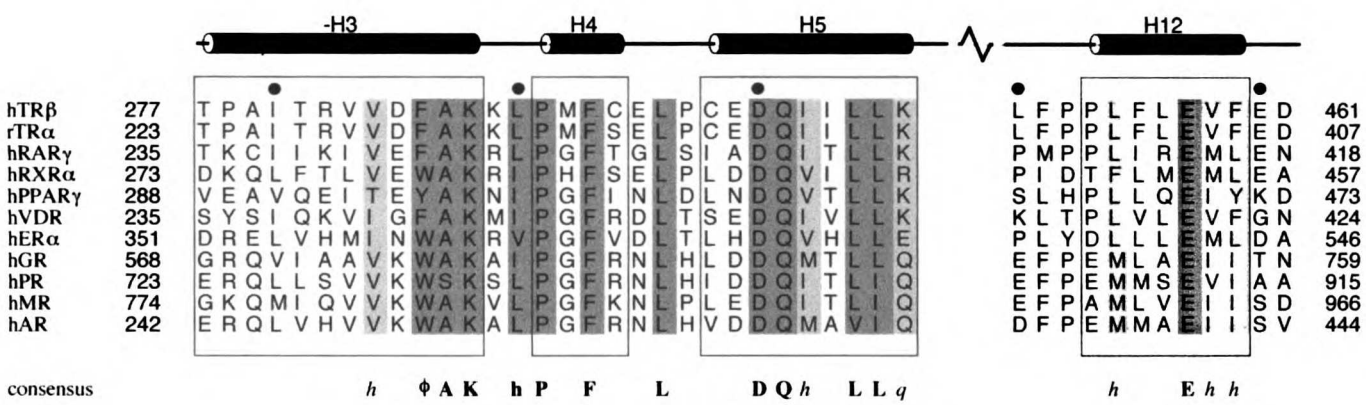
Figure 2-13 Sequence alignment of LBD helices 3, 4, 5 and 12

Nuclear receptor sequences are shown for LBD regions that interact with GRIP1. Residues representing the "nuclear receptor signature sequence", ΦAKxhPxFxxLxxxDQxxhh, are on dark background. Functionally conserved residues, always hydrophobic (h) or strong polar (q), are shaded light gray. The borders for helices H3, H4, H5, and H12 for the TRβ LBD are shown as a rough guide for other receptors.

Figure 2-13 Sequence alignment of LBD helices 3, 4, 5 and 12



four fold compared to wild type TRβ LBD, whereas the affinity for NR-box 3 was marginally increased (Figure 2.14) . We conclude that the higher affinity of NR-box 2 for TRβ LBD reflects a favourable interaction between positively charged residues N-terminal to the LxxLL motif and negatively charged residues at the C-terminus of the TRβ LBD.

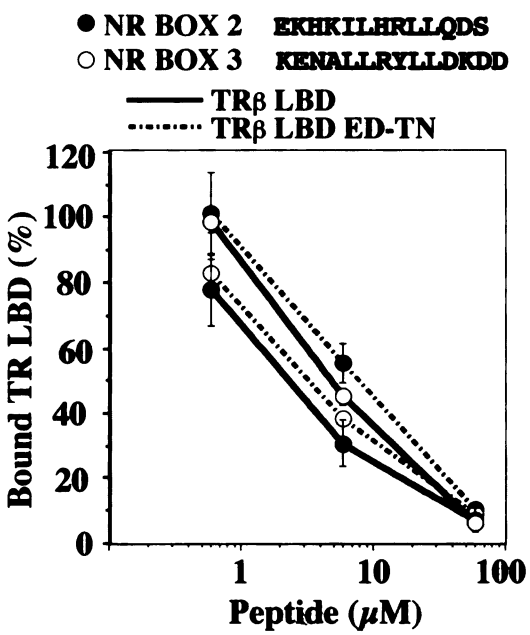
A role for helix stabilization?

One explanation for poor competition by the NR-box 3 hexapeptide LLRYLL for TRβ LBD (Figure 2.3) may be the high energy of α-helix formation by short peptides. In fact, in the TRβ LBD:NR-box 2 complex, the peptide helix appears to be stabilized by two helix-capping interactions: one between the side chain of TRβ K288, which interacts with the free carbonyl groups of L694 of peptide A; and one between the side chain of TRβ E457 in H12, which interacts with the backbone amides of I689 and L690 in both peptide complexes. The TRβ residues K288 and E457 are highly conserved among nuclear receptors, and mutations in these or homologous residues eliminate the interaction of TRβ or the estrogen receptor (ER) with p160 coactivators, perhaps implying that capping interactions are functionally important in coactivator binding (Danielian, White et al. 1992; Barettino, Vivanco Ruiz et al. 1994; Durand, Saunders et al. 1994; Tone, Collingwood et al. 1994; Henttu, Kalkhoven et al. 1997; Feng, Ribeiro et al. 1998).

Figure 2-14 The C-terminus of the TR LBD discriminates between NR-boxes

Labeled, in vitro translated TRβ LBD (solid line) or TRβ LBD ED-TN (dashed line) (10 nM) was incubated with 1.6 μM glutathione agarose-bound GST-NID3^{-} in the presence of 10 μM T_3 and increasing concentrations of peptides containing NR-box 2 EKHKILHRLLQDS (solid circle), or NR-box 3 KENALLRYLLDKDD (open circle). The amount of bound receptor is relative to the amount of retained receptor in the absence of peptide. The data represent the average and standard deviation of three independent experiments.

Figure 2-14 The C-terminus of the TR LBD discriminates between NR-boxes



Interaction specificity

The features of the interface in the TRβ LBD that interacts with the NR-box 2 LxxLL motif are highly conserved within the nuclear receptor family (Figure 2.13) [Bourguet, 1995 #4032; Renaud, 1995 #4573; Wagner, 1995 #4348; Brzozowski, 1997 #4883;Wurtz, 1996 #4774; Williams, 1998 #5323] the hydrophobic groove is present in all known structures of receptor LBDs, and many residues within and surrounding the groove were identical or, as demonstrated by mutational analysis, functionally conserved across the family (Danielian, White et al. 1992; Barettino, Vivanco Ruiz et al. 1994; Durand, Saunders et al. 1994; Tone, Collingwood et al. 1994; Renaud, Rochel et al. 1995; Henttu, Kalkhoven et al. 1997; Feng, Ribeiro et al. 1998). The conservation of the interacting surfaces may explain the broad specificity of members of the p160 coactivator family for nuclear receptors.

However, it is apparent that receptors interact selectively with particular p160 family members, and with individual NR-boxes within a given coactivator. In fact, functional and interaction studies revealed nuclear receptor-selective interactions with various p160 coactivators (Ding, Anderson et al. 1998; Voegel, Heine et al. 1998). To identify the determinants of selectivity, we compared the interaction of GRIP1 with TRβ and the glucocorticoid receptor (GR), another nuclear receptor family.

Similar to TRβ and TRβ LBD, GR bound the GRIP1 NID in a ligand-dependent manner and failed to bind to NR-box 1; in contrast to TRβ, however, GR preferred NR-box 3 over NR-box 2 (Figure 2.15) . These *in vitro* results parallel those obtained in

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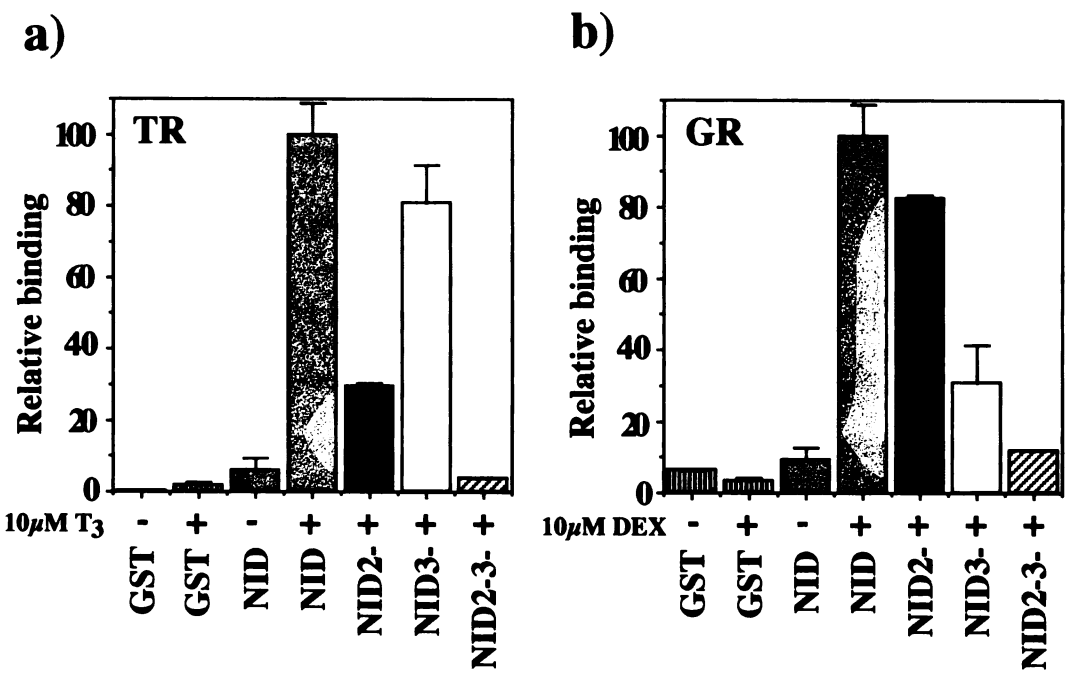
Figure 2-15 GR and TR interact preferentially with different GRIP1 NR-boxes

Labeled, *in vitro* translated TRβ (a) or rat GR (b) (10 nM) was incubated with 1.6 μM glutathione agarose-bound GST (vertically striped), GST-NID (gray) or the NID variants NID2$^-$ (black), NID3$^-$ (white) and NID2$^-$3$^-$ (diagonally striped). Binding of the receptors

Figure 2-15 GR and TR interact preferentially with different GRIP1 NR-boxes



to these GRIP1 fragments was performed in the absence (–) or presence (+) of 10 μM T_3 (TR) or 10 μM DEX (GR). The amount of bound receptor is normalized to fraction bound.

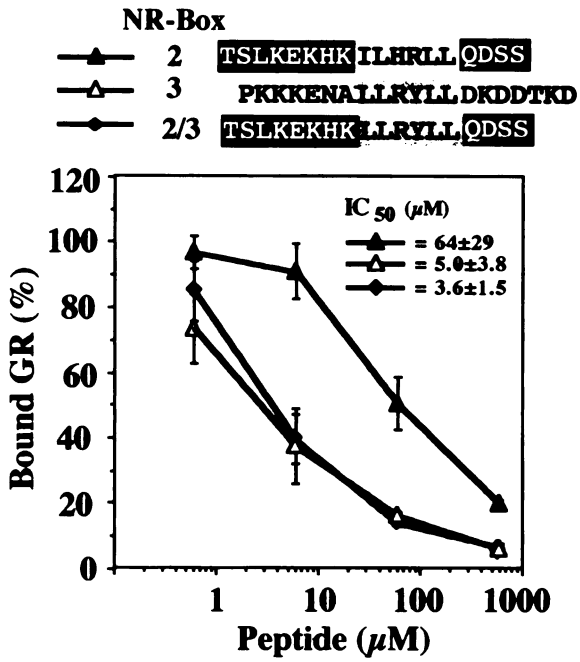
yeast 2-hybrid assays *in vivo*, using mutants of NR-boxes 2 or 3 in the context of full length GRIP1 (Ding, Anderson et al. 1998). Consistent with this observation, a peptide containing NR-box 3 competed the GR NID interaction with higher affinity than a peptide containing NR box 2 (Figure 2.16) . Surprisingly, NR-box 3 peptide PKKKENALLRYLLDKDDTKD inhibited the interaction of the NID with GR with the similar efficiency (IC_{50} = 5±3.8 μM) as the interaction with TRβ (IC_{50} = 2.9±1.0 μM), whereas the affinity of NR-box 2 peptide TSLKEKHKILHRLLQDSS for GR (IC_{50} = 64±29 μM) was about 100-fold lower than for TRβ (IC_{50} = 0.4±0.1 μM) (compare Figure 2.3, competition of the interactions between TRβ or TRβ LBD and NID or NID3$^-$ by these peptides was comparable (data not shown)).

In the case of TRβ LBD, preference of NR-box 2 over 3 was dependent on the adjacent sequences of NR-box 2, as demonstrated by a chimeric peptide containing the hydrophobic LLRYLL motif of NR-box 3 in the context of the adjacent sequences of the NR-box 2 motif (Figure 2.14). In contrast, the chimeric peptide and the NR-box 3 peptide competed the interaction of the NID with GR comparably (Figure 2.16). Thus, the preference of GR for NR-box 3 is not specified by the adjacent sequences, and instead appears to reflect differences in the motif itself (NR-box 2, ILHRLL; NR-box3, LLRYLL).

Figure 2-16 Preferential interaction of GR with NR-box 3 is not specified by sequences adjacent to the motif

In vitro translated, labeled GR (10 nM) was incubated with 1.6 μM glutathione agarose-bound GST-NID3$^-$ in the presence of increasing concentrations of NR-box 2 peptide TSLKEKHKILHRLLQDSS (solid triangle), NR-box 3 peptide PKKKENALLRYLLDKDDTKD (open triangle), or a chimeric peptide TSLKEKHKLLRYLLQDSS bearing the LLRYLL motif of NR-box 3 with the adjacent sequences from NR-box 2 (gray diamond). The binding assay was performed in the presence of 10 μM DEX. The amount of bound receptor is relative to the amount of retained receptor in the absence of peptide. The data and IC_{50} values represent the average and standard deviation of three independent experiments.

Figure 2-16 Preferential interaction of GR with NR-box 3 is not specified by sequences adjacent to the motif



The differences in affinity and preference for NR-box 2 or 3 by GR and TRβ LBD likely reflect receptor-specific structural differences in the proximity of the coactivator interaction surface. For example, GR lacks the C-terminal negatively charged residues (Figure 2.13) that in TRβ LBD stabilized the interaction with NR-box 2, and substitution of these TRβ residues by the corresponding GR residues resulted in decreased affinity for NR-box 2 (Figure 2.14). However, whereas this TRβ mutant interacted with only slight preference (2-fold) for NR-box 3 over NR-box 2, the affinity of GR for NR-box 2 is about 6-fold lower than for NR-box 3, suggesting that in GR additional components disfavour interaction with NR-box 2. Besides the absence of negatively charged residues C-terminal of helix 12, another obvious difference between GR and TRβ is the so-called F-domain, which extends the C-terminus of the GR well beyond the end of H12 and is absent in TRβ. Although the structure of the GR LBD is not known, the structure of the closely related PR LBD (Williams and Sigler 1998) indicates that the F-domain may restrict the coactivator interaction surface and displace the N-terminal residues of the NR-box. In any case, the comparison of GR and TRβ demonstrates that at least two components, the LxxLL motif and the sequences flanking the motif, can be used differentially to generate distinct specific interactions with the p160 coactivator GRIP1.

DISCUSSION

Nuclear receptor–NR box interactions

The interaction of nuclear receptors with coactivators of the p160 family depends on the presence of NR-boxes that contain the hydrophobic LxxLL motif. As demonstrated by the structure of the TRβ LBD:NR-box 2 peptide complex, the hydrophobic residues of this LxxLL motif interact with a conserved, hydrophobic surface on the LBD of nuclear receptors. High affinity interaction depends on the integrity of the entire motif, but affinity and specificity of the interaction can be modulated by sequences adjacent to the motif. In addition to the role of these structural elements in establishing specific interactions, a region of GRIP1 outside of the NID contributes to the overall affinity of the GR:NR-box 2 interaction (H. Hong and MRS, unpublished results).

In the case of the TRβ LBD, negatively charged residues at the C-terminus of the receptor stabilized the interaction with NR-box 2, probably by interacting with positively charged residues adjacent to the LxxLL motif. In the absence of this stabilizing interaction, as in the case of GR or the TR ED:TN mutant, NR-box 3 interacted with higher affinity than NR-box 2, and the preference for NR-box 3 was independent of the adjacent sequences. *In vivo* studies have identified several nuclear receptors for which preferential binding to NR-box 2 correlates with acidic residues C-terminal of helix 12(Ding, Anderson et al. 1998; Voegel, Heine et al. 1998) . Notably, however, even closely related receptors, such as GR and the mineralocorticoid receptor, differ in their NR-box preference (Ding, Anderson et al. 1998). Conceivably, the multiple NR-boxes found in p160 coactivators may enable diversity in the interaction with different nuclear receptors.

What is AF-2?

The AF-2 transcriptional activation function requires helix H12 at the C-terminus of the receptor LBD, as mutations within this helix eliminate AF-2 activity (Danielian, White et al. 1992; Barettino, Vivanco Ruiz et al. 1994; Durand, Saunders et al. 1994; Tone, Collingwood et al. 1994) . Fusions of H12 to the Gal4 DNA binding domain were reported to activate transcription, leading to the suggestion that H12 alone corresponds to AF-2 [Barettino, 1994 #3340; Durand, 1994 #3870;Saatcioglu, 1997 #4809]. However, point mutations in H12 had very different effects on activation in the Gal4 fusion context than in the intact LBD (Saatcioglu, Lopez et al. 1997). Subsequently, it was inferred from comparisons of the structure of the unliganded hRXRα LBD with that of the liganded TR and RAR LBDs that ligands induced dramatic conformational changes, affecting especially the orientation of H12 (Bourguet, Ruff et al. 1995; Renaud, Rochel et al. 1995; Wagner, Apriletti et al. 1995). On that basis, helix H12 was proposed as a ligand-dependent switch that forms, in its functional configuration, a surface for coactivator recognition or binding(Moras and Gronemeyer 1998).

We have demonstrated that residues of H12 are indeed directly involved in coactivator binding. However, in agreement with mutational studies implicating other parts of the receptor LBDs in AF-2 activity and interaction with coactivators(Renaud, Rochel et al. 1995; Henttu, Kalkhoven et al. 1997; Feng, Ribeiro et al. 1998; Norris *et al.* 1998) our structural analysis reveals that GRIP1 interacts with helices H3, H4, H5 and H12 of the TRβ LBD. Thus, AF-2 corresponds to the full coactivator interaction surface, and not to H12 alone.

Ligand effects on LBD structure and function

With respect to the LBD, agonists are ligands that support its interaction with coactivators, presumably by promoting formation of the coactivator interaction surface described here. Antagonists, such as the glucocorticoid receptor ligand RU486, or the estrogen receptor ligand raloxifene, do not support LBD interactions with p160 coactivators (Hong, Kohli et al. 1997; Norris, Fan et al. 1998). Comparison of the estradiol- and raloxifene-bound structures of the ERα LBD reveals strikingly different positions of helix 12 (Brzozowski, Pike et al. 1997). In the ER:raloxifene complex, H12 packs against the hydrophobic residues of helices H3 and H5 in a configuration remarkably similar to that of the NR-box 2 peptide of GRIP1 with the agonist-bound TR LBD. Thus, raloxifene precludes the interaction of ER with coactivators in two ways: first, eliminating the part of the coactivator interaction surface that involves H12, and second, occluding with H12 the remaining part of the coactivator interaction surface.

As many nuclear receptor antagonists, like raloxifene, appear structurally related to agonists that have been modified by a long "central extension", it seems likely that antagonists will commonly function by interfering with positioning of H12 in the coactivator interface. It is intriguing to consider whether the position of H12 seen in the ER:raloxifene complex will also prove to be general. In that structure, H12 contacts H3 and H5 through a hydrophobic surface (LLxxML) similar to the p160 LxxLL motif. The corresponding face of H12 among other nuclear receptors, while less similar to LxxLL, also contains bulky hydrophobic residues, raising the possibility that the H12:H3/H5 interface may be commonly adopted when the "agonist conformation" is precluded.

Common protein-protein interfaces

The structure of the TRβ LBD:GRIP1 interaction shows striking similarities with the other three described protein-protein interfaces in transcriptional regulatory complexes: p53:MDM2(Kussie *et al.* 1996), VP16:$TAF_{II}31$(Uesugi, Nyanguile et al. 1997) and CREB:CBP(Radhakrishnan *et al.* 1997). These complexes share a common theme: the interaction of an amphipathic α-helix containing a conserved hydrophobic motif with a complementary, hydrophobic surface. As with GRIP1, a functional interaction appears to require specific hydrophobic residues; for example, Fxxhh has been proposed as a motif that interacts selectively with $TAF_{II}31$(Uesugi, Nyanguile et al. 1997).

Consistent with a close fit between the hydrophobic surfaces at the interfaces, even conservative replacements of the hydrophobic residues at the interface typically compromise the affinities of the interactions. However, some factors, such as $TAF_{II}31$ and MDM2(Lu and Levine 1995; Martin *et al.* 1995; Thut *et al.* 1995; Kussie, Gorina et al. 1996; Uesugi, Nyanguile et al. 1997), may interact functionally with more than one hydrophobic motif, suggesting that precise complementarity of the interacting surfaces may be less important for these interactions.

Based on the shared similarities in the structures of these four complexes and the potential for cross-interaction involving different hydrophobic motifs, we tested whether the GRIP1 NID could interact with $TAF_{II}31$ or MDM2. GRIP1 NID chimeras in which the NR-box 2 LxxLL motif was replaced by the corresponding VP16 (FxxAL) or p53 (FxxLW) sequences were also tested. In no case did we observe GRIP1 NID interactions with $TAF_{II}31$ or MDM2 (data not shown). Clearly, the presence of the correct motif alone is not sufficient for high affinity binding, consistent with the notion that specificity in the interactions of these two common structural features is determined by a combination of parameters that include adjacent sequences, and perhaps more remote influences on protein

conformation. The use of multiple determinants in defining common interactions provides mechanisms both for flexible assembly and for specificity.

Combinatorial regulation

Transcriptional regulatory complexes are dynamic structures that can assemble differentially from the mixture of components available in different cell and response element contexts, but are nevertheless sufficiently precise in structure that they produce specific regulatory consequences. We suggest that the combinatorial assembly of these complexes is achieved in part by complementary interactions between two simple structural features, an amphipathic α-helix and a hydrophobic groove, at least one of which is present on each interacting component. Interestingly, the critical contacts at the interaction interfaces are restricted to a surprisingly small area. Thus, it may eventually prove possible to mimic or disrupt those interactions with small molecules.

In the nuclear receptor LBDs, formation of the coactivator-interacting hydrophobic groove is ligand-dependent, and coactivator specificity is achieved by contributions from several distinct determinants: the α-helical LxxLL motif, the residues immediately adjacent to the motif, and as shown by others, sequences outside the nuclear receptor interaction domain. This strategy for building specificity into protein:protein interactions, through differential contributions of various features within a common structural interface, may prove to be general for many components of transcriptional regulatory complexes.

MATERIALS AND METHODS

Vectors. hTRβ LBD (His_6 E202-D405) was expressed from a pET28a (Novagen)-based construct (A. Shiau, unpublished). pET GRIP1 563-767His_6 (encoding the NID) and pGEX-4T1 GRIP1 563-767His_6 (encoding GST-NID)were constructed by cloning a GRIP1 *Bam* HI - *Xho* I fragment derived from pGEX-2TK GRIP1 563-1121 (H. Hong et al., in preparation) into the *Bam* HI-*Xho* I sites of pET23a or pGEX-4T1His_6; the latter was generated by exchanging the *Xho* I-*Bsa* AI fragment of pGEX-4T1 against a His_6-tag-containing *Xho* I-*Nae* I fragment of pET23a (Novagen). NID mutants were generated by PCR or single stranded mutagenesis and confirmed by sequencing.

Protein expression. NID and GST-NID derivatives were expressed in HB101 (37°C, 1mM IPTG added at OD_{600}=0.7, induced 4 h). hTRβ LBD was expressed in BL21DE3 (14°C, 1mM IPTG added at OD_{600}=0.7, induced 24 h).

Protein purification. Cell lysis: For GRIP1 derivatives, sonication buffer (20 mM TrisHCl pH 8.0, 100 mM NaCl, 10% glycerol, 0.1 mM PMSF and protease inhibitors (Complete, EDTA free, Boehringer Mannheim)). For TRβ LBD, 50 mM sodium-phosphate pH 8.0, 300 mM NaCl, 10% glycerol, 25 mM β mercaptoethanol, 0.1 mM PMSF. Purification: Freeze-thaw, incubate with 0.1 mg/ml lysozyme (20 min, 0°C); sonicate, clear lysate (Ti45, 36000 rpm, 1h, 4°C). For GST-NID, load lysate on Talon resin (Clontech) equilibrated in sonication buffer; elution with imidazole gradient (12-100 mM) yields 95% pure protein. For NID, same Talon purification as above; protein elutes at 12 mM and at 40-70 mM imidazole; 12 mM fraction was chromatographed on MonoQ (50 mM TrisHCl pH7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and protease inhibitors; NID flowed through (>95% pure) and was concentrated by ultrafiltration. For TRβ LBD, same Talon purification as above, except in sodium

phosphate buffer, eluted with 12-300 mM imidazole gradient. Isolation of liganded (3,3',5-triiodo-L-thyronine (T_3; Sigma)) receptor using TSK-phenyl HPLC (TosoHaas, Philadelphia) was as described(Apriletti, Baxter et al. 1995); yield was 9.5 mg/L bacterial culture. For crystallization, hTRβ LBD was diluted into 20 mM Hepes pH7.4, 3 mM DTT and 0.1 μM T_3 using NAP columns (Pharmacia), and concentrated to 9 mg/ml by ultrafiltration (Millipore UFV2BGC10).

Glutathione agarose binding. Imidazole was removed by NAP gel filtration (Pharmacia); protein concentrations determined by BioRad assay . Wild type or mutant GST-NID was incubated with glutathione agarose (1h, 4°C) in binding buffer (sonication buffer + 1 mM DTT, 1 mM EDTA, 0.01% NP-40). Beads were washed with >20 volumes of binding buffer, diluted in binding buffer with 20% glycerol to 40% final bead concentration; aliquots were frozen in liquid nitrogen and stored at -70°C. Loaded beads were analyzed by SDS PAGE using BSA standard (Pierce) to ensure equal concentrations of bound proteins.

In vitro transcription-translation. rGR was cloned as a *Bam* HI/*Eag* I fragment into the BamHI/Bgl II site of pSG5 (Stratagene) after blunting the *Eag* I and *Bgl* II ends with Klenow. pSG5 hTRβ was constructed as described (Feng, Ribeiro et al. 1998). pSG5 rGR, pSG5 hTRβ and pET28a hTRβLBD were used to express ^{35}S-labeled rGR, hTRβ, and hTRβ LBD in a coupled reticulocyte lysate (TNT; Promega). Translations were performed in the presence and absence of 10 μM dexamethasone (GR), or 10 μM T_3 (hTRβ and hTRβ LBD). Expression of rGR, hTRβ, and hTRβ LBD yielded 5-50, 20-100 and 100-250 ng/μl reaction, respectively, as computed from the amount of incorporated ^{35}S methionine.

Interaction assay. 50 μl of 20% bead suspension containing 1.6 or 4.0 μM GST NID or derivatives was incubated with 0.2-2 μl lysate containing labeled rGR, hTRβ, or hTRβ

LBD; final concentration of receptors was ~10 nM; binding was independent of lysate concentration. Binding (4°C under rotation, 2 h) was in binding buffer + 20 μg/ml BSA, with or without 10 μM hormone. For competition experiments, peptides were added before receptors, but altering the order of addition did not affect the results (data not shown), confirming that the reactions reach equilibrium. Beads were washed (4°C, five times, 200 μl binding buffer), proteins eluted (10 μl 2x SDS loading buffer), subjected to SDS-PAGE, and the fraction of bound receptor determined (Molecular Dynamics PhosphorImager, Storm 860) as percentage relative to input (in direct binding studies) or as percentage relative to bound receptor in the absence of ligand (in competition studies).

Peptides. Peptides (from UCSF Biomolecular Resource Center, Research Genetics, or generously provided by Tularik Inc) were HPLC purified and analyzed by mass spectroscopy. Concentrations were determined spectroscopically using the tyrosine signal (ε_{276} = 1450 $M^{-1}cm^{-1}$) or by amino acid analysis (Protein and Nucleic Acid Facility, Stanford University Medical Center).

Crystallization. Several peptides containing NR-box 2 were tested in crystallization trials with TRβ LBD; crystallization was achieved with peptide KHKILHRLLQDSS. The complex was prepared by mixing 9 mg/ml TRβ LBD (20 mM HEPES pH 7.4) with 14 mM peptide (0.4 mM ammonium acetate pH 4.72), at a 1:2 ratio, 0°C, 1h. Crystals were obtained (2 d, 4°C) by hanging drop vapor diffusion from a drop (1.5 μl of TRβ LBD:NR-box 2 peptide complex and 0.5 μl 15% PEG 4K, 200 mM sodium citrate pH 4.9) suspended above a reservoir containing 10% PEG 4K, 100 mM ammonium acetate, and 50 mM sodium citrate (pH 5.6). After 1 h, 0.2μl of 10^{-3} to 10^{-5} dilutions of microcrystals in reservoir buffer were introduced for nucleation. Crystals were space group $P3_121$ (a=95.2 Å, c=137.6 Å) and contained 2 molecules each of TRβ LBD and NR-box 2 peptide.

Structural analysis. Crystals were soaked overnight in a drop composed as described above plus 10% sucrose; then transferred serially, 5 min each, to drops containing 10% PEG4K, 100 mM ammonium acetate, and 50 mM sodium citrate (pH 5.6), 10% sucrose, excess NR-box 2 peptide, and increasing concentrations of xylotol (2% to 13% by 2% steps). Crystals were then suspended in a nylon loop attached to a mounting pin, and flash frozen (liquid nitrogen). Data were measured using Cu Kα radiation (R-axis generator, 50 kV, 300 mA, 0.3 mm collimator, Ni filter); reflections were recorded using an R-Axis II detector, integrated with Denzo, and equivalent reflections scaled using Scalepack (Otwinowski and Minor 1997). Possible rotation function solutions were calculated using normalized amplitudes in AMORE from a model of TRβ LBD with ligand omitted; translation function solutions were determined using TFFC for the two rotation solutions with the highest correlation coefficients (Project 1994). After rigid body refinement of the TRβ LBD molecules, electron density maps were calculated, and positive difference density used to fit the iodine atoms of T_3. The iodine atoms were modeled as a rigid body, and refined with the TRβ LBD against a maximum likelihood target with strict non-crystallographic symmetry using CNS (A.T.Brunger, personal communication)(Adams *et al.* 1997). 2FoFc and FoFc electron density maps showed interpretable density, related by the non-crystallographic symmetry operator, near H12 of both molecules of the TRβ LBD. Several helical models, in both orientations, were fit to the density using O (Jones, Zou et al. 1991) . The electron density was best modeled as an short α-helix, using observed side chain density to assign sequence and chain direction. Refinement included positional refinement with CNS interspersed with manual rebuilding, monitored using the free-R factor. A flat bulk solvent correction, and an overall anisotropic B-factor correction, were applied. The refined model consists of residues K211-P254 and V264-D461 of monomer 1, residues K211-P254 and G261-D461 of monomer 2, residues 688-KILHRLLQDSS-698 of peptide 1, and residues 688-KILHRLLQD-696 of peptide 2.

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Conclusion

Underlying every fundamental biological problem is a basic question: given a limited repetoire of genes, how does life specify a vast number of distinct states? Formally, each distinct state could derive from the actions of a unique set of genes. But such a scheme places an onerous burden on evolution, since adaptation would require the creation of a new solution to suit each problem. The apparent conservation of gene products and fundamental cellular processes across organisms argues that evolution proceeds differently: having solved a problem once, life feels no compulsion to revisit it, and shamelessly copies its work forward (Vale and Fletterick 1997; Kimura *et.al.*, 1997; Lin *et. al.*, 1997).

The development of methods for cloning sheep and mice from mitotic cells, by emphasizing that all cells contain the same set of genes, implies a contrasting biological economy (Wilmut *et al.*, 1997; Wakayama *et al.*, 1998). Evidently, the distinct patterns of regulation that distinguish skin from bone, or mouse from man, can be produced simply through combinations and recombinations of a limited set of genes. But if the combinatorial regulation model theoretically provides enough adaptability to empower evolution, and enough flexibility to enable development and differentiation, crucially the mechanisms of that regulation remain unclear.

Writing about the proliferin (plfG) composite regulatory element, Yamamoto and colleagues introduced terminology and concepts which remain useful in describing and analysing mechanisms for combinatorial regulation (Yamamoto *et al*, 1992, and references therein). Combinatorial regulation requires junction points for the integration of information from multiple signalling systems. Here the signals interact and combine, leading to a particular regulatory consequence. Presuming that the signals function in multiple contexts imputes two paradoxical features to the interaction: as the desired regulatory outcome must be exact, the interaction needs to be precise; but to allow a

multiplicity of outcomes, the interaction must be transient. A viable mechanism of combinatorial regulation, then, must resolve the paradox at the molecular level.

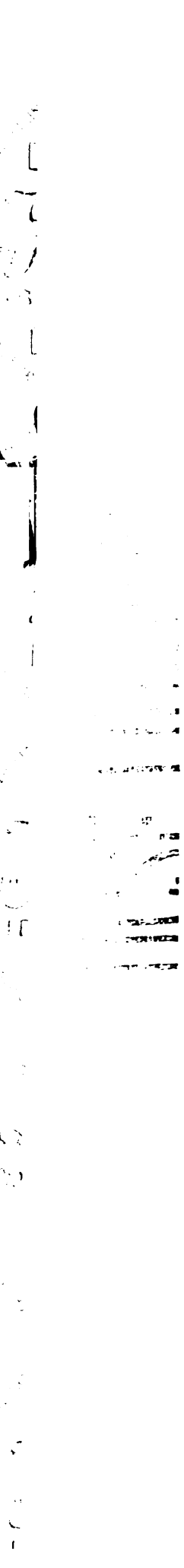
The plfG response element, described by Yamamoto and colleagues, illustrates the requirements of combinatorial regulation. A response element, a DNA sequence which binds signalling factors to modulate transcriptional initiation from a neighboring promoter, functions as a junction point when the range of regulatory outcomes produced are distinct from those of the individual factors or of the simple sum. The plfG response element, by either activating or repressing transcriptional initiation depending on the subunit composition of the multimeric factor AP-1, qualifies as a junction point, or composite regulatory element. The ability of the plfG composite element to produce opposite transcriptional effects relies discrete specificity domains within each factor. In the case of the glucocorticoid receptor (GR), specificity lay in its unique amino-terminal domain, which distinguishes it from the closely related, but inactive, mineralocorticoid receptor (MR). In the case of the AP-1 multimer, the specificity was localized to a few amino-acids in the basic region of the conserved DNA recognition helix, which determinate the regulator outcome: activation (c-Jun/c-Jun or c-Jun/Fra-1) or repression (c-Jun/c-Fos). Presumably, these specificity domains form an interaction surface, apparently satisfying the constraints of sensitivity and flexibility to produce opposite transcriptional effects using at least four factors (GR, MR, c-Jun/c-Jun, c-Jun/c-Fos).

Though not as well studied as the plfG element, the p160 coactivator : nuclear receptor interaction also appears to act as a junction point. Subtle differences in activity between closely-related factors are characteristic of combinatorial regulation: the three individual members of the p160 family of coactivators interact promiscuously with the nuclear receptors, but superimposed on that promiscuity are receptor-specific differences in the interaction. Functional consequence of those differences remains unclear, but (insert

reference to Holly here), while presumably utilizing the same surface. Further examples of functional differentiation establish the p160 coactivator : nuclear receptor interaction as a junction point.

Several studies, including our own, identified the specificity elements of the nuclear receptor LBD and p160 coactivator that permit diversity of response. Directed mutagenesis of the surface of the nuclear receptor LBD identified several hydrophobic and charged residues that reduced both coactivator interaction and transcriptional activation; as these residues clustered together, it was presumed the residues identified the receptor interaction surface, or AF-2 activation domain (Feng *et al.* 1998; Darimont *et al.*, 1998). In the p160 coactivators, the specificity domain was localized using peptides to short, discrete regions containing a central (h)LxxLL motif flanked on either side by variable residues; these were termed NR-boxes (Ding *et al.*, 1997; Darimont *et al.*, 1998). Crystallographic studies of the thyroid hormone receptor LBD in complex with a peptide of NR-box 2 of GRIP-1 confirmed a direct interaction between the two elements.

The homology in the specificity domains within nuclear receptor and p160 coactivator families implied possible redundancy; but, as in the case of the plfG composite element, studies revealed distinct preferences among individual nuclear receptors for particular NR-boxes. With structural information on the complex that had been lacking in the case of the plfG composite element, conclusions could be drawn and tested regarding the origin of specificity. One source might be the variable residues flanking the (h)LxxLL of the NR-box, which could make receptor-specific contacts in addition to the (h)LxxLL motif. Apparently, this model accounts for the observed specificity of the TR, which can discriminate between NR-box peptides based on flanking sequences. A second source might be alterations in the AF-2 domain, achieved primarily through the angle of H12 (e.g. PR vs TR), which could produce a complementary surface that favored a particular



(h)LxxLL sequence. This model accounts for the observed specificity of the GR, which discriminates between related NR-boxes 2 and 3 using solely the (h)LxxLL motif.

The versatility of the p160 coactivator: nuclear receptor interaction is quite remarkable. Strikingly, very similar interactions observed in other transcriptional regulatory complexes; does this structural convergence say something about its desirability as a framework for combinatorial regulation? For example, the restriction of the NR-box to a few residues facilitates evolution, by allowing adaptation to occur through relatively few changes; the moderate (low micromolar) affinity for the interaction assures a transient interaction; the NR box possess the innate modularity characteristic of regulatory domains. But as yet unaddressed is the fundamental question of plasticity - how and whether the NR box adapts to distinct receptors to convey specificity; how and whether the AF-2 of nuclear receptors tolerates other regulatory proteins; how and whether other signals, such as phosphorylation, modulate the interaction to achieve greater diversity. With evidence of plasticity, and further supporting exmaples of functional differentiation between p160 coactivators, then the argument of combinatorial regulation from the p160 coactivator : nuclear receptor will be convincing.

Appendix One

Related Publications

The following was originally published as "Preliminary crystallographic studies of the ligand-binding domain of the thyroid hormone receptor complexed with triiodothyronine," by M.E. McGrath *et. al.*, in the March 25, 1994 issue of the *Journal of Molecular Biology* (**237**(2): 236-239.) The paper describes the crystallization protocol for Form I of the rat α TR LBD, the structure of which is further discussed in Chapter One of this thesis.

Preliminary Crystallographic Studies of the Ligand-binding Domain of the Thyroid Hormone Receptor Complexed with Triiodothyronine

Mary E. McGrath[1]†, Richard L. Wagner[2], James W. Apriletti[3], Brian L. West[3] V. Ramalingam[1], John D. Baxter[3]‡ and Robert J. Fletterick[1]‡

[1]*Department of Biochemistry and Biophysics*

[2]*Graduate Group in Biophysics*

[3]*Metabolic Research Unit, University of California San Francisco, San Francisco, CA 94143, U.S.A.*

A truncated, recombinant form of the thyroid hormone receptor, including the hormone binding domain, has been co-crystallized with the hormone T_3. The crystals are monoclinic, most likely space group $P2_1$ with two molecules per asymmetric unit and cell dimensions $a = 63{\cdot}6$ Å, $b = 80{\cdot}8$ Å, $c = 100{\cdot}9$ Å and $\beta = 92{\cdot}1°$. The crystals diffract to only medium resolution and decay rapidly in the X-ray beam using laboratory sources. By contrast, high resolution, high-quality data are obtained using synchrotron radiation in conjunction with cryocrystallography.

Keywords: crystallization; thyroid hormone; receptor; cryocrystallography; synchrotron

Thyroid hormones are iodinated dityrosine derivatives which direct events in development, differentiation, growth and other physiological processes (Eberhardt *et al.*, 1980; Glass & Holloway, 1990; Oppenheimer & Samuels, 1983). The particular response is elicited when 3,5,3′-L-triiodothyronine (T_3§) binds to chromatin-associated nuclear receptors which are capable of binding DNA and modulating gene transcription (Oppenheimer & Samuels, 1983). Thyroid hormone receptors belong to a superfamily of hormone receptors which includes the glucocorticoid, estrogen, retinoic acid, and vitamin D receptors. These proteins function by regulating transcription in a ligand-responsive fashion. The ligands which bind can be quite dissimilar in structure (Evans, 1988).

Two different thyroid hormone receptor genes (α and β) have been identified (Koenig *et al.*, 1988; Murray *et al.*, 1988; Thompson *et al.*, 1987*a*). The predicted amino acid sequences of the thyroid hormone receptors and other steroid/thyroid receptor superfamily members strongly suggests that they possess a domain structure. These domains include an amino-terminal domain, a conserved cysteine-rich DNA binding domain, a hydrophilic hinge region, and, at the carboxyl end, a ligand-binding domain (Evans, 1988). Functional evidence for this domain structure has been obtained from gene transfer studies using genes that express either hybrid proteins in which domains of the various receptors have been swapped, or mutations or deletions affecting the selected receptor functions such as DNA and hormone binding (Thompson & Evans, 1989; Zhang *et al.*, 1991).

Mutations within the ligand-binding domain of the thyroid hormone receptor reveal several interesting properties of the receptor, such as a repressor function, dimer and heterodimer formation functions, and modulation of DNA binding (Zhang *et al.*, 1991). Patients with thyroid resistance syndromes frequently have mutations in the ligand-binding domain which diminish T_3 binding and cause the receptor to function as a dominant negative regulator (Mixson *et al.*, 1992). Approximately half of these patients have an attention deficit-hyperactivity disorder (Hauser *et al.*, 1993). This first example of a psychiatric disorder resulting from a genetic abnormality has generated additional interest in the structure of the thyroid hormone receptor.

Formation of heterodimers of the T_3 receptor and other transcription factors such as the retinoid X receptor, appears to be crucial for transcriptional

† Present address: Khepri Pharmaceuticals, Inc., 260 Littlefield Avenue, South San Francisco, CA 94080, U.S.A.

‡ Authors to whom correspondence should be addressed.

§ Abbreviations used: T_3, 3,5,3′-L-triiodothyronine; TR-LBD, thyroid hormone receptor containing the hinge region and ligand-binding domain; MPD, 2-methyl-2,4-pentamediol.

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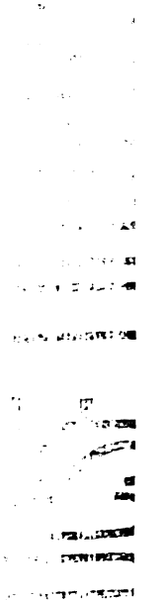

regulation by the T_3 receptor (Marks *et al.*, 1992; Yu *et al.*, 1991). Subregions of the ligand-binding domains have been identified which appear essential for dimerization (Lee *et al.*, 1992; O'Donnell *et al.*, 1991; Spanjaard *et al.*, 1991). Also, a heptad repeat is present in the ligand-binding domain which has been postulated to form a leucine zipper-like structure (Forman *et al.*, 1989).

An X-ray structure has been reported for the DNA-binding domain of the glucocorticoid receptor (Luisi *et al.*, 1991). No structures for the ligand-binding domains for the steroid/thyroid type receptors have been determined. We have found that a truncated thyroid hormone receptor containing the hinge region and ligand-binding domain (TR-LBD) can be stably produced in bacteria (Apriletti *et al.*, unpublished results), which encouraged us to pursue the X-ray structure of this receptor fragment. This paper describes preliminary studies which should culminate in a high-resolution structure of TR-LBD. A structural description of this domain will provide a picture of the hormone-binding site and much additional information regarding transcriptional control in the steroid/thyroid hormone systems.

Expression of TR-LBD and purification to greater than 99·5% was performed (Apriletti *et al.*, unpublished results). TR-LBD is an N-terminally truncated form of rat thyroid hormone receptor α (Thompson *et al.*, 1987*b*) comprising residues 122 to 410. This moiety lacks the DNA binding domain, but includes the entire hinge region and entire hormone-binding domain. Purified TR-LBD has a ligand specificity and T_3 binding affinity (K_D = 0·06 nM) equivalent to that of the full-length rat thyroid hormone receptor α (Apriletti *et al.*, unpublished results).

The TR-LBD purification procedure ensures that TR-LBD is stoichiometrically bound with T_3 (Apriletti *et al.*, unpublished results). To maintain full saturation of TR-LBD with T_3, and to prepare the complex for crystallization, the hormone-bound TR-LBD is concentrated and desalted in an Amicon Centricon-10 microconcentrator (McGrath *et al.*, 1989), using 10 mM Hepes (pH 7·0), 3·0 mM DTT, and 1·0 nM T_3. For crystallization experiments involving other ligands, the other ligand was substituted for T_3 in the final purification step and in the microconcentrator step.

Factorial crystallization screening trials (Jancarik & Kim, 1991) were carried out for TR-LBD bound to T_3 (M_r = 33,000) using hanging-drop vapor diffusion (with 1 μl protein solution, 1 μl precipitant solution and a 0·5 ml reservoir using silanized coverslip: McPherson, 1982) at 17°C. TR-LBD is not stable at 4°C and is stored at −80°C, where it maintains its avidity for hormone and its crystallizability for approximately two to three months. Crystals were obtained in condition 21 of the screening trials (Jancarik & Kim, 1991) and conditions were then optimized. Wedge-shaped crystals are reproducibly obtained at 17°C with 16% to 22% 2-methyl-2,4-pentanediol (MPD), (Aldrich Chemical Co., Inc.), 0·2 M ammonium acetate and 0·1 M sodium cacodylate (pH 6·7) over a period of three days. The best crystals have a limiting dimension of approximately 100 μm and are obtained at a protein concentration of 7 mg/ml in the presence of 3 mM DTT. The particular nucleation *versus* crystal growth characteristics of this system result in comparable crystal quality over a relatively broad range of MPD concentration; a related phenomenon is that we are unable to improve our crystals by any crystal seeding protocol (e.g. dilutional microseeding with 10^3 to 10^6 dilutions of crushed crystals, or macroseeding with repetitively washed (5×) small crystals) (Stura & Wilson, 1990). Crystals suitable for diffraction are obtained by using 10 μl hanging drops.

Significant non-isomorphism is observed for similarly prepared crystals, and although early experiments showed that approximately 25% of the crystals did not diffract X-rays to a detectable extent, inclusion of 3 mM DTT, as described above, ameliorated this problem. The diffraction limit using conventional laboratory sources (Rigaku Rotaflex generator at 50 kV × 60 mA, with Ni-filtered CuKα X-rays collimated to 0·3 mm) appears to be between 3·0 and 3·2 Å. The crystals decay rapidly in the X-ray beam. Cryocrystallography (equipment purchased from Molecular Structure Corp., Houston, TX) on an *R*-axis image plate system (Rigaku RTP500 generator operating at 50 kV × 180 mA, with a graphite monochromator and collimated to 0·3 mm) was attempted in which the MPD-derived crystals were placed in a blocked nitrogen stream, then flash frozen at −160°C and maintained in that stream for data collection. MPD acts as a suitable cryosolvent, eliminating the need to treat the crystals with additional cryoprotectants. The diffraction limit of the crystals appears to be unaffected by freezing. The mosaicity for the diffraction maxima was approximated as 0·7°.

A data set for TR-LBD bound to T_3 was collected at the Stanford Synchrotron Radiation Laboratory (beamline 7-1) using cryocrystallographic methods and the MAR image plate detector. The crystals diffracted to at least 2·05 Å under these conditions (60 s frames, wavelength = 1·08 Å), and no significant decay was observed during data collection. Frames subtending 1·0° of oscillation were collected for extended times in order to record the weaker, high resolution data. Two 90° batches of data were collected on each of two crystals. Contiguous collection of a hemisphere of data was precluded because the crystals were suspended in a nichrome loop (Teng, 1990) (prior to freezing) which resulted in metal diffraction when the loop approached a parallel orientation with respect to the incident X-ray beam. Recent optimization of glass loops for cryocrystallography (M. Stowell, C.I.T., personal communication) has eliminated this inconvenience.

The MOSFLM (Nyborg & Wonacott, 1977) data reduction package (as modified by A. Leslie, Imperial College) was used at UCSF along with CCP4 programs (CCP4, 1979) to process the raw

regulation by the T_3 receptor (Marks *et al.*, 1992; Yu *et al.*, 1991). Subregions of the ligand-binding domains have been identified which appear essential for dimerization (Lee *et al.*, 1992; O'Donnell *et al.*, 1991; Spanjaard *et al.*, 1991). Also, a heptad repeat is present in the ligand-binding domain which has been postulated to form a leucine zipper-like structure (Forman *et al.*, 1989).

An X-ray structure has been reported for the DNA-binding domain of the glucocorticoid receptor (Luisi *et al.*, 1991). No structures for the ligand-binding domains for the steroid/thyroid type receptors have been determined. We have found that a truncated thyroid hormone receptor containing the hinge region and ligand-binding domain (TR-LBD) can be stably produced in bacteria (Apriletti *et al.*, unpublished results), which encouraged us to pursue the X-ray structure of this receptor fragment. This paper describes preliminary studies which should culminate in a high-resolution structure of TR-LBD. A structural description of this domain will provide a picture of the hormone-binding site and much additional information regarding transcriptional control in the steroid/thyroid hormone systems.

Expression of TR-LBD and purification to greater than 99·5% was performed (Apriletti *et al.*, unpublished results). TR-LBD is an N-terminally truncated form of rat thyroid hormone receptor α (Thompson *et al.*, 1987*b*) comprising residues 122 to 410. This moiety lacks the DNA binding domain, but includes the entire hinge region and entire hormone-binding domain. Purified TR-LBD has a ligand specificity and T_3 binding affinity (K_D = 0·06 nM) equivalent to that of the full-length rat thyroid hormone receptor α (Apriletti *et al.*, unpublished results).

The TR-LBD purification procedure ensures that TR-LBD is stoichiometrically bound with T_3 (Apriletti *et al.*, unpublished results). To maintain full saturation of TR-LBD with T_3, and to prepare the complex for crystallization, the hormone-bound TR-LBD is concentrated and desalted in an Amicon Centricon-10 microconcentrator (McGrath *et al.*, 1989), using 10 mM Hepes (pH 7·0), 3·0 mM DTT, and 1·0 nM T_3. For crystallization experiments involving other ligands, the other ligand was substituted for T_3 in the final purification step and in the microconcentrator step.

Factorial crystallization screening trials (Jancarik & Kim, 1991) were carried out for TR-LBD bound to T_3 (M_r = 33,000) using hanging-drop vapor diffusion (with 1 μl protein solution, 1 μl precipitant solution and a 0·5 ml reservoir using silanized coverslip: McPherson, 1982) at 17°C. TR-LBD is not stable at 4°C and is stored at −80°C, where it maintains its avidity for hormone and its crystallizability for approximately two to three months. Crystals were obtained in condition 21 of the screening trials (Jancarik & Kim, 1991) and conditions were then optimized. Wedge-shaped crystals are reproducibly obtained at 17°C with 16% to 22% 2-methyl-2,4-pentanediol (MPD), (Aldrich Chemical Co., Inc.), 0·2 M ammonium acetate and 0·1 M sodium cacodylate (pH 6·7) over a period of three days. The best crystals have a limiting dimension of approximately 100 μm and are obtained at a protein concentration of 7 mg/ml in the presence of 3 mM DTT. The particular nucleation *versus* crystal growth characteristics of this system result in comparable crystal quality over a relatively broad range of MPD concentration; a related phenomenon is that we are unable to improve our crystals by any crystal seeding protocol (e.g. dilutional microseeding with 10^3 to 10^6 dilutions of crushed crystals, or macroseeding with repetitively washed (5×) small crystals) (Stura & Wilson, 1990). Crystals suitable for diffraction are obtained by using 10 μl hanging drops.

Significant non-isomorphism is observed for similarly prepared crystals, and although early experiments showed that approximately 25% of the crystals did not diffract X-rays to a detectable extent, inclusion of 3 mM DTT, as described above, ameliorated this problem. The diffraction limit using conventional laboratory sources (Rigaku Rotaflex generator at 50 kV × 60 mA, with Ni-filtered CuKα X-rays collimated to 0·3 mm) appears to be between 3·0 and 3·2 Å. The crystals decay rapidly in the X-ray beam. Cryocrystallography (equipment purchased from Molecular Structure Corp., Houston, TX) on an *R*-axis image plate system (Rigaku RTP500 generator operating at 50 kV × 180 mA, with a graphite monochromator and collimated to 0·3 mm) was attempted in which the MPD-derived crystals were placed in a blocked nitrogen stream, then flash frozen at −160°C and maintained in that stream for data collection. MPD acts as a suitable cryosolvent, eliminating the need to treat the crystals with additional cryoprotectants. The diffraction limit of the crystals appears to be unaffected by freezing. The mosaicity for the diffraction maxima was approximated as 0·7°.

A data set for TR-LBD bound to T_3 was collected at the Stanford Synchrotron Radiation Laboratory (beamline 7-1) using cryocrystallographic methods and the MAR image plate detector. The crystals diffracted to at least 2·05 Å under these conditions (60 s frames, wavelength = 1·08 Å), and no significant decay was observed during data collection. Frames subtending 1·0° of oscillation were collected for extended times in order to record the weaker, high resolution data. Two 90° batches of data were collected on each of two crystals. Contiguous collection of a hemisphere of data was precluded because the crystals were suspended in a nichrome loop (Teng, 1990) (prior to freezing) which resulted in metal diffraction when the loop approached a parallel orientation with respect to the incident X-ray beam. Recent optimization of glass loops for cryocrystallography (M. Stowell, C.I.T., personal communication) has eliminated this inconvenience.

The MOSFLM (Nyborg & Wonacott, 1977) data reduction package (as modified by A. Leslie, Imperial College) was used at UCSF along with CCP4 programs (CCP4, 1979) to process the raw

data and produce the list of observed structure factor amplitudes. The crystals belong to the monoclinic crystal system with lattice parameters $a=63{\cdot}6$ Å, $b=80{\cdot}8$ Å, $c=100{\cdot}9$ Å and $\beta=92{\cdot}1°$. We used XPREP, part of the SHELLXTL program package (Siemens Inc., Madison WI), to analyze the reduced data for space group determination. No reflections which violate the 2-fold screw symmetry have intensities greater than 3σ. We reduced the data in space group $P2$, though we cannot eliminate $P2_1$. The calculated unit cell volume is approximately 518,000 Å^3. Crystal density was measured using a Ficoll gradient (Westbrook, 1985) and found to be approximately 1·08 g/ml. Thus, we expect two molecules per asymmetric unit, with a crystal packing parameter (Matthews, 1968), V_m, of 3·7 Å^3/dalton. Conversely, the estimated solvent content of this crystal form is 67%.

The R_{sym} value of the 2·05 Å data set is 0·086 (0·095 when Friedel mates were merged) for 126,000 measurements of 34,000 independent reflections. We are currently searching for suitable heavy-atom derivatives.

We have crystallized LBD in complexes with other thyroid hormones and derivatives including T4 (thyroxine), triac (3,3′,5-triiodothyroacetic acid), isopropyl T2 (3,5-diiodo,3′-isopropylthyronine), and dimit (3,5-dimethyl-3′-isopropylthyronine), an analog with no iodine atoms. The co-crystals are similarly obtained at the same pH, and over the same broad range of MPD concentration as described above, and are all morphologically indistinguishable from the LBD-T3 co-crystals. Preliminary diffraction data with the TR-LBD dimit-bound co-crystals showed that they are isomorphous with the TR-LBD-T3 unit cell. Since many of the hormones and analogs differ in the number of iodine atoms, we will attempt to use one or more of the co-crystals as derivatives for phasing. In addition to molecular averaging and solvent flattening, anomalous scattering from the iodine atoms in LBD-T3 can be helpful in resolving phase ambiguities with conventional single isomorphous replacement data. We have also initiated a collaboration to attempt *ab initio* phasing using the iodine anomolous data with multiple anomalous diffraction measurements (MAD: Hendrickson, 1985).

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The following was originally published as "A natural transactivation mutation in the thyroid hormone beta receptor: impaired interaction with putative transcriptional mediators," by T.N. Collingwood, *et al.* in the January 7, 1997 issue of the *Proceedings of the National Academy of Sciences U S A* (**94**(1): 248-53). The paper identifies a residue, L454 in the human β TR LBD, which, when mutated, results in impaired interaction of the TR with p160 coactivators. As the homologous residue in the rαTR LBD lies of the surface of the TR LBD, the authors suggests that residue may participate directly in the interaction with coactivators. The role of L454 in binding NR box 2 of the p160 coactivator GRIP1 is discussed in Chapter Two of this thesis.

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A natural transactivation mutation in the thyroid hormone β receptor: Impaired interaction with putative transcriptional mediators

(resistance to thyroid hormone/hormone-dependent transactivation/coactivator)

T. N. COLLINGWOOD*†, O. RAJANAYAGAM*†, M. ADAMS*, R. WAGNER‡, V. CAVAILLÈS§, E. KALKHOVEN§, C. MATTHEWS*, E. NYSTROM¶, K. STENLOF¶, G. LINDSTEDT¶, L. TISELL¶, R. J. FLETTERICK‡, M. G. PARKER§, AND V. K. K. CHATTERJEE*

*Department of Medicine, University of Cambridge, Level 5, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, United Kingdom; ‡Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448; ¶Sahlgren's University Hospital, University of Gothenburg, S-41345 Gothenburg, Sweden; and §Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

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ABSTRACT **The syndrome of resistance to thyroid hormone is characterized by elevated serum free thyroid hormones, failure to suppress pituitary thyrotropin secretion, and variable peripheral refractoriness to hormone action. Here we describe a novel leucine to valine mutation in codon 454 (L454V) of the thyroid hormone β receptor (TRβ) in this disorder, resulting in a mutant receptor with unusual functional properties. Although the mutant protein binds ligand comparably to wild-type receptor and forms homo- and heterodimers on direct repeat, everted repeat, or palindromic thyroid response elements, its ability to activate transcription via these elements is markedly impaired. The hydrophobic leucine residue lies within an amphipathic α-helix at the carboxyl terminus of TRβ and the position of the homologous residue in the crystal structure of TRα indicates that its side chain is solvent-exposed and might interact with other proteins. We find that two putative transcriptional mediators (RIP140 and SRC-1) exhibit hormone-dependent association with wild-type TR. In comparison, the interaction of this natural mutant (L454V) and artificial mutants (L454A, E457A) with RIP140 and SRC-1 is markedly reduced. Furthermore, coexpression of SRC-1 is able to restore the transcriptional activity of the L454V mutant receptor, indicating that the interaction of this residue with accessory proteins is critical for transcriptional activation. Finally, the occurrence of the L454V mutation in resistance to thyroid hormone, together with impaired negative regulation of the thyroid-stimulating hormone α promoter by this mutant, suggests that the amphipathic α-helix also mediates hormone-dependent transcriptional inhibition, perhaps via interaction with these or other accessory factors.**

Resistance to thyroid hormone (RTH) is characterized by elevated serum-free thyroid hormones, failure to suppress pituitary thyroid-stimulating hormone (TSH) secretion, and variable refractoriness to hormone action in peripheral tissues. Following the observation that familial RTH was tightly linked to the thyroid hormone receptor β (TRβ) gene locus (1), a growing number of β-receptor mutations have been identified in kindreds with this disorder (2, 3). In keeping with other members of the nuclear receptor superfamily, the TR is organized into distinct functional domains (4). The carboxyl-terminal (D/E) region of TR mediates hormone binding and nuclear localization functions and also contains sequences that mediate homodimeric TR interactions and the formation of heterodimers with the retinoid X receptor (RXR) (5). All the RTH mutations described to date localize to the D/E domains and the majority cluster within two hot spots (6, 7). Their properties have provided valuable insights into structure–function relationships in the receptor. Most mutant receptors exhibit reduced hormone binding (6–8), whereas their ability to localize to the nucleus is not impaired (8). With a few exceptions (9–11), mutant receptors retain the ability to form homodimers and they all heterodimerize with the RXR (9, 12). These properties correlate with the location of mutation clusters outside regions that are involved in heterodimerization (5) and which are less important for hormone binding (13).

The D/E domains of TR also confer the ability to regulate target gene transcription in a hormone-dependent manner. A nine amino acid sequence at the receptor carboxyl terminus has been shown to be essential for this ligand-inducible transactivation function (AF-2) and is deleted in v-erbA, the oncogenic counterpart of TR (14). This region is highly conserved among nuclear receptors and mutational analyses in a number of them have demonstrated its importance in transactivation (15–20). Here we describe a natural mutation substituting valine for leucine at codon 454 within this sequence motif in TRβ. Although it retains DNA binding, hormone binding, and dimerization functions, this mutant receptor transactivates target genes poorly and is a powerful dominant negative inhibitor of wild-type (WT) receptor action. Comparison with the crystal structure of the ligand-binding domain of TRα (21) indicates that this leucine residue is solvent-exposed with the potential to interact with other proteins. To test this hypothesis, we examined the interaction of WT and mutant TRs with two putative transcriptional mediators, RIP140 and SRC-1, which have recently been isolated (22–24). RIP140 is a 140-kDa protein that exhibits hormone-dependent binding to the estrogen receptor (ER) and was identified by Far Western blot analysis. SRC-1 is a 125-kDa protein that was identified by its binding to the progesterone receptor in the presence of ligand in a yeast two-hybrid assay system.

MATERIALS AND METHODS

Clinical and Genetic Studies. The proband (AJ) presented at the age of 20 with palpitations and was noted to have a goiter

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Abbreviations: RTH, resistance to thyroid hormone; TSH, thyroid-stimulating hormone; TR, thyroid hormone receptor; TRβ, thyroid hormone receptor β; RXR, retinoid X receptor; WT, wild type; β-gal, β-galactosidase; ER, estrogen receptor; mER, mouse estrogen receptor; RAR, retinoic acid receptor; TRE, thyroid response element.

and raised serum thyroid hormones. Following initial treatment with carbimazole she underwent thyroid surgery and was free of symptoms for 10 years. The goiter and tachycardia recurred, necessitating radioiodine treatment, following which she developed hypothyroidism requiring thyroxine replacement. Despite 200 μg thyroxine daily, the TSH response to TRH was exaggerated, rising from a basal value of 35 mu/liter to a peak of 275 mu/liter 30 min after TRH. The TSH response was reduced (95 mu/liter), but preserved following T3 administration (120 μg/4 days), suggesting a diagnosis of resistance to thyroid hormone. The daughter of the proband (CJ) had low weight, tachycardia, tremor, and a small goiter at birth. A diagnosis of neonatal thyrotoxicosis was made initially and carbimazole treatment instituted. Symptoms improved during infancy and thyroxine therapy to control her goiter was discontinued at puberty. Current symptoms include palpitations, muscle weakness, and sensitivity to bright light. The sister (ES) of the index case showed abnormal thyroid function on biochemical screening and was asymptomatic for many years, but recently developed a persistent tachycardia. Serum-free T4 and serum-free T3 levels were measured with fluoroimmunometric assays using Delfia technology (Wallac, Milton Keynes, U.K). TSH measurements were performed using a sensitive "second generation" assay (Wallac). Current thyroid function tests in the affected individuals are as follows: proband (AJ) free T4 75 pmol/liter (normal 9–20), free T3 16 pmol/liter (normal 3–7.5), and TSH 0.4 mu/liter (normal 0.4–4); daughter (CJ) free T4 49 pmol/liter, free T3 14 pmol/liter, and TSH 1.3 mu/liter; sister (ES) free T4 54 pmol/liter, free T3 18 pmol/liter, and TSH 1.7 mu/liter.

Leukocyte DNA from each affected individual was extracted using standard techniques. Coding exons of the human (h)TRβ gene were amplified using PCR and then sequenced directly as described (7). The presence of a receptor mutation was verified by at least two independent PCRs and sequencing reactions in each individual. The nomenclature used to describe the receptor mutation conforms to a consensus statement from the First Workshop on Thyroid Hormone Resistance (25).

Hormone- and DNA-Binding Assays. Mutations were introduced into the receptor by site-directed mutagenesis of the hTRβ1 cDNA in M13mp18 as described (9) and verified by sequencing. WT and mutant receptor proteins were synthesized by coupled *in vitro* transcription and translation with rabbit reticulocyte lysate (TNT, Promega) and the T3-binding affinity of each was determined using a filter-binding assay (7).

Receptor binding to DNA was determined by electrophoretic mobility-shift assay using *in vitro* translated receptors and oligonucleotide duplexes corresponding to an optimized palindromic thyroid response element (TRE), a direct thyroid response element TRE from the malic enzyme gene promoter, and an everted repeat TRE from the chicken lysozyme gene (9). The ability of WT and mutant TR-RXR heterodimers to bind T3 was tested using a reverse gel mobility-shift assay. The procedure was identical to the conventional assay except that the *in vitro* translated receptor was labeled with [^{35}S]methionine and incubated with a non-labeled oligonucleotide duplex in the presence or absence of 1 nM ^{125}I-labeled T3. Receptor-associated ^{125}I-T3 was selectively detected by autoradiography in the presence of a ^{35}S filter.

Transfection Assays. WT and mutant receptor function was assayed by cotransfection with reporter genes into JEG-3 human choriocarcinoma cells as described (9). WT and mutant hTRβ cDNAs were expressed using a vector containing the Rous sarcoma virus (RSV) enhancer and promoter. RSVCAT, driving the expression of chloramphenicol acetyltransferase, was used as control expression vector. PALTKLUC, MALTKLUC, and F2TKLUC contain two copies of a palindromic TRE or a single copy of a direct repeat or everted repeat TRE, respectively, upstream of the thymidine kinase promoter and luciferase cDNA (9). TSHαLUC contains the 5′-flanking region of the human TSHα-subunit gene from −846 to +44 bp, coupled to luciferase (9). The internal control plasmid Bos–β-galactosidase (β-gal), containing the human elongation factor 1α gene promoter driving β-gal, was used to correct for variations in transfection efficiency. SRC-1 was amplified from a human B-cell cDNA library with primers based on the previously reported SRC-1 sequence (24) and cloned into a eukaryotic expression vector (pSG5).

Protein–Protein Interaction Assays. Fusion proteins containing the ligand-binding domain of WT of mutant receptors linked to glutathione *S*-transferase were expressed in *Escherichia coli* and purified as described (18). The vector containing RIP140 has been described elsewhere (23). The SRC-1 cDNA described above was cloned into pBluescript SK+. [^{35}S]Methionine-labeled proteins were synthesized by coupled *in vitro* transcription and translation of each cDNA (TNT system, Promega). Aliquots of immobilized fusion protein were incubated with either *in vitro* translated ^{35}S-labeled RIP140 or SRC-1 proteins in PD buffer (50 mM Tris·HCl/0.1 M KCl/0.14 M NaCl/0.5% Nonidet P-40/10% glycerol, pH 8.0) in the presence or absence of 5 μM ligand for one hr at room temperature, then washed four times with NETN buffer (20 mM Tris·HCl/0.1 M NaCl/1 mM EDTA/0.5% Nonidet P-40, pH 8.0) and analyzed by SDS/PAGE (18). Gels were Coomassie stained to check for equal loading of fusion protein, then exposed to autoradiography.

RESULTS

Genetic Analyses. Direct sequencing of exon 10 of the TRβ gene in the proband (AJ) indicated that she was heterozygous for a single nucleotide substitution (1645 TTG to GTG), which corresponded to a leucine to valine mutation at codon 454 (L454V) in the predicted amino acid sequence. Exons 4–9, encoding all but the first eight amino acids of the β receptor, were also sequenced in this individual and no other abnormalities were detected. Subsequent analyses showed that her daughter (CJ) and sister (ES) were also heterozygous for this mutation, demonstrating concordance between the presence of a receptor defect and biochemical abnormalities that are typical of RTH.

Hormone and DNA Binding. To determine its functional consequences, the L454V mutation was introduced into the WT TRβ1 cDNA and mutant protein generated by coupled transcription and translation *in vitro*. Hormone-binding assays showed comparable binding of ^{125}I-T3 to WT and mutant receptor proteins and Scatchard analysis indicated that their ligand-binding affinities were similar [Mean and SEM of at least three determinations: WT, $K_a = 1.8 \times 10^{10}\ M^{-1}$ (±0.3); L454V, $K_a = 1.2 \times 10^{10}\ M^{-1}$ (±0.1)].

Previous studies (26, 27) indicate that TR can bind to DNA either as a homodimer or as a heterodimer with RXR on different TRE configurations. Accordingly, the DNA-binding properties of WT and mutant receptors were studied using everted repeat (F2), palindromic (PAL), and direct repeat (ME) TREs in the presence of hRXRα (Fig. 1*a*). Under these conditions the L454V mutant formed homodimer and heterodimer complexes on F2 and PAL and heterodimers on the direct repeat (MAL) comparably with WT. The WT and mutant homodimer complexes were equally attenuated following ligand binding (28), whereas the TR–RXR complexes were quantitatively unaffected but showed a small change in mobility. Recent studies have shown that in some contexts, heterodimerization can induce allosteric changes, which preclude the binding of ligand to either receptor partner (29, 30).

†T.N.C. and O.R. contributed equally to this work and should both be considered first authors.

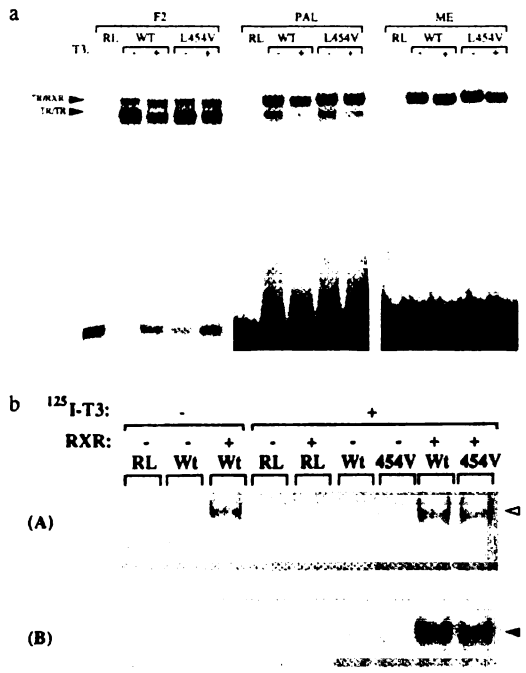

FIG. 1. (*a*) DNA binding and dimerization of WT and mutant (L454V) receptors. Electrophoretic mobility-shift assays show the formation of TRβ homodimers and TR/RXRα heterodimers (arrowheads) in the presence (+) or absence (−) of 10 nM T3 on everted repeat (F2), palindromic (PAL), and direct repeat (ME) configurations of TRE. (*b*) Both WT and mutant (454V) receptors retain the ability to bind T3 when bound to DNA as a heterodimer with RXRα. ^{35}S-labeled receptors were incubated with unlabeled malic enzyme TRE oligonucleotide duplexes in the presence or absence of ^{125}I-labeled T3. (A) Formation of heterodimers with RXRα by ^{35}S-labeled TRβ. (B) A ^{125}I-selective exposure of the same gel showing retention of ligand binding by both WT and L454V TR-RXR heterodimers. RL denotes reticulocyte lysate control.

To confirm that the L454V receptor mutant was still able to bind ligand while complexed with RXR, reverse electrophoretic mobility-shift assays were performed using MAL TRE. [^{35}S]Methionine-labeled TR and RXR were incubated with unlabeled TRE in the presence or absence of ^{125}I-T3 and analyzed by PAGE (Fig. 1*b*). Fig. 1*b* (*Upper*) displays the formation of [^{35}S]methionine-labeled TR–RXR complexes (lanes 3, 8, and 9). Exposure of the same gel to detect ^{125}I-T3 (Fig. 1*b Lower*) showed that both WT and mutant heterodimer complexes were labeled with radioligand (lanes 8 and 9).

Functional Activity and Dominant Negative Inhibition. The function of WT and L454V mutant receptors was studied in transient transfection assays using a reporter gene (TSHαLUC) containing the negatively regulated human TSHα promoter (Fig. 2*a*). Inhibition of TSHαLUC by mutant receptor was impaired, exhibiting a right-shifted profile compared with WT, with maximal inhibition attained at higher T3 concentrations. This is particularly evident when the significant background inhibition of TSHαLUC activity in RSV-CAT-transfected cells, which may be mediated by endogenous TR, is taken into account.

Similar studies were performed using the positively regulated direct repeat (MALTKLUC), everted repeat (F2TKLUC), and palindromic (PALTKLUC) TRE-containing reporter genes. In comparison with significant hormone-dependent activation seen with WT on MAL and F2 (Fig. 2 *b* and *c*), the L454V mutant was unable to induce significant reporter gene activity, even with saturating concentrations of T3. With a palindromic TRE (Fig. 2*d*) the mutant receptor retained some hormone-dependent activation potential, but this was markedly reduced (~30%) compared with its WT counterpart.

The dominant negative potential of the L454V receptor mutant was tested. Either WT receptor alone or WT plus an equal amount of L454V mutant was cotransfected with reporter genes (TSHα, MAL, F2, PAL) at low (0.05 nM TSHα; 1nM MAL/F2/PAL) or high (10 nM TSHα; 1000 nM MAL/F2/PAL) T3 concentrations, and luciferase activity was measured. In the presence of mutant receptor, induction of all three reporter genes by WT receptor was markedly inhibited (MAL, 31%; F2, 50%; PAL, 32% versus 100% with WT alone) at low T3 concentrations (Fig. 2 *f–h*). Even at higher T3 concentrations, the L454V mutant continued to exert a significant inhibitory effect (MAL, 40%; F2, 34%; PAL, 68%). The mutant receptor also exerted a dominant negative effect with the negatively regulated TSHα promoter at low T3 concentrations, which was reversed at higher levels (Fig. 2*e*).

Location of Homologous Leucine in Structure of the rTRα Ligand-Binding Domain and Protein–Protein Interaction Assays. The crystal structure of the ligand-binding domain of TRα has recently been elucidated (21), enabling us to examine the position of the residue homologous to Leu-454 of TRβ, Leu-400. As shown in Fig. 3*a*, this hydrophobic residue is not directly involved in interaction with ligand, in keeping with its preserved hormone-binding affinity. Furthermore, the residue is solvent accessible (Fig. 3*b*), suggesting that it could be involved in protein–protein interactions.

To test this proposal, we examined the interaction of WT and mutant TRs with RIP140, a protein known to interact with the ER. In addition to the natural L454V mutant, we tested artificial mutations of this (L454A) and an adjacent charged residue (E457A, E457D). We have shown previously that these mutations also have little effect on ligand binding (18). However, the L454A mutant exhibits negligible ligand-dependent transactivation and the transcriptional activity of the E457A mutant is also markedly attenuated. The E457D mutant does retain modest transcription activation function (18). In a protein–protein interaction assay the ligand-binding domain of WT TRβ exhibited strong T3-dependent interaction with ^{35}S-labeled RIP140, comparable to hormone-dependent interaction of this protein with ER (Fig. 4*a*). In comparison, the ligand-dependent binding of the transactivation mutants (L454A, L454V, E457A) to RIP140 was abolished or markedly attenuated, whereas the E457D mutant retained weak association with this protein. The relative interaction of different mutants with RIP140 was too low to be reliably quantitated.

In a further study, the association of these mutant receptors with the putative transcriptional coactivator SRC-1 was also examined (Fig. 4*b*). As with RIP140, the L454A and E457A mutations severely reduced ligand-dependent interaction of the receptor with SRC-1 (2 ± 1% and 6 ± 2%, respectively, relative to WT), in accordance with the minimal transcriptional activity of these mutants. The L454V mutant, which is less transcriptionally impaired than L454A and E457A, retained moderate interaction with SRC-1 (39 ± 4%). In contrast to its binding to RIP140, interaction of the E457D mutant with SRC-1 was comparable to that of WT receptor (96 ± 13%).

Effect of Coexpressed SRC-1 on Activity of WT and L454V Mutant Receptors. As the L454V mutant receptor exhibits a significantly reduced affinity for SRC-1 *in vitro*, we hypothesized that enhancing the level of SRC-1 expression might alleviate its loss of transactivation function. WT or L454V mutant receptors were tested with MAL or PAL reporter genes together with increasing amounts of cotransfected SRC-1 expression vector, in the absence and presence of a

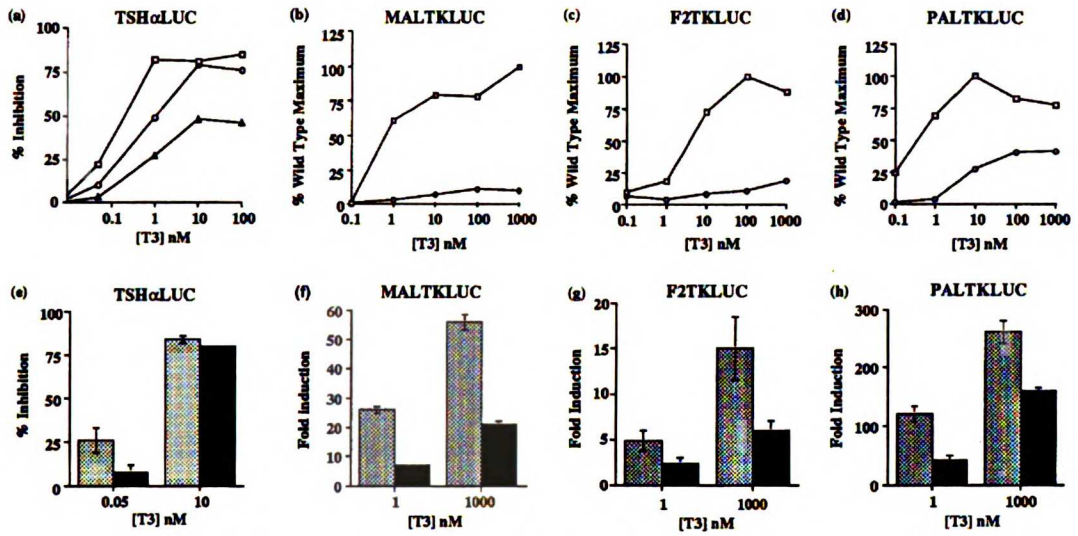

FIG. 2. (*a–d*) Modulation of reporter gene expression by WT (□) or L454V mutant receptors (○). Six-well plates of JEG-3 cells were transfected with 2 μg (TSHαLUC, MALTKLUC, PALTKLUC) or 4 μg (F2TKLUC) of reporter gene, 100 ng of receptor expression vector, and 200 ng of Bos–β-gal. Following incubation with T3, luciferase activity was normalized for β-gal activity. Hormone-dependent inhibition of TSHαLUC is expressed relative to values in non-T3 treated cells. Inhibition of TSHαLUC in control cells transfected with 100 ng RSVCAT is also shown (Δ). Activation of MAL/PAL/F2 reporters by L454V is expressed as a percentage of the maximal induction by WT receptor. The results shown are the mean of at least three independent transfections in triplicate and the SEM was less than 5% (TSHαLUC) or 10% (MAL/F2/PAL). (*e–g*) Dominant negative activity of L454V mutant receptor. JEG-3 cells were transfected with reporter and reference plasmids as in *a–d* and 100 ng of WT receptor expression vector together with an equal amount of RSVCAT (shaded) or L454V mutant (solid) expression vectors. Inhibition or induction of reporter activity was calculated following incubation with low or high T3 concentrations as indicated. The results are the mean of at least three transfections in triplicate.

maximal T3 concentration. With MALTKLUC, hormone-dependent activation by WT receptor was not significantly enhanced by SRC-1 (Fig. 5*a*). In contrast, the transcriptional activity of the L454V mutant was markedly augmented, increasing from 7% to 47% of WT levels with incremental amounts of SRC-1. The addition of SRC-1 had little effect on basal reporter activity with either WT or mutant receptor. With the PALTKLUC reporter, cotransfection of SRC-1 increased hormone-dependent activation by the WT receptor. However, activation by the L454V mutant receptor was also progressively augmented by SRC-1, restoring its function to levels attained by WT receptor (Fig. 5*b*). Again, coexpressed SRC-1 had negligible effects on basal promoter activity, except at the highest level of added SRC-1.

DISCUSSION

The three affected individuals in this kindred showed biochemical abnormalities which are characteristic of RTH, with markedly elevated serum FT4 and FT3 levels and a failure to suppress TSH secretion. The proband's sister (ES) had been virtually asymptomatic and was therefore classified as a case of generalized resistance. In contrast, the proband (AJ) and her daughter (CJ) had experienced thyrotoxic symptoms, suggesting predominant pituitary resistance. The coexistence of generalized resistance and pituitary resistance phenotypes in a single family with the same receptor mutation highlights the variability of clinical features in RTH and supports previous observations that indicate that generalized resistance and pituitary resistance are differing manifestations of a single genetic disorder (7, 31).

When the transcriptional and hormone-binding properties of natural mutant receptors associated with RTH are considered, three types of mutants have been described (32): type I mutants exhibit reduced transactivation in keeping with their altered ligand binding, but higher T3 concentrations elicit full activation; type II mutants have disproportionately reduced transactivation relative to their altered ligand binding at both low and high T3 concentrations; type III mutants neither bind ligand nor transactivate. The activation profiles of the L454V mutant (Fig. 2) are most consistent with type II properties. A possible cause of impaired transactivation is an alteration in receptor DNA binding or dimerization functions. For example, another natural transactivation mutant (R429Q) exhibits impaired homodimer formation on some TREs (9, 11). However, normal homo- and heterodimer formation by the L454V mutant (Fig. 1*a*) does not favor this hypothesis. These assays also demonstrate that L454V mutant homodimer complexes are attenuated in the presence of T3 (Fig. 1*a*) and that heterodimers retain ^{125}I-T3 binding (Fig. 1*b*). The latter suggests that allosteric changes in the mutant receptor following DNA binding, which might preclude ligand occupancy (29, 30) are also an unlikely explanation for its altered transactivation.

Significantly, we and others (9, 33) have identified a number of other natural RTH mutations (P453A, P453H, P453S, P453T) with impaired transactivation, involving the proline residue that immediately precedes the leucine at codon 454, as well as seven additional residues at the TRβ carboxyl terminus. This sequence motif can be delineated in a number of other nuclear receptors (15), with striking conservation of hydrophobic and negatively charged residues. Studies have shown that mutation of a negatively charged glutamic acid residue in the ER (15), retinoic acid receptor (RARα) (19), or TR (16–18, 34) impairs transactivation with little effect on ligand binding. A double mutation (L543A/L544A) of hydrophobic residues in mouse ER (mER) (15) also disrupts transactivation and the L454V mutation we have described is homologous to the proximal leucine residue in mER. Together, these findings underscore the importance of this proximal hydrophobic res-

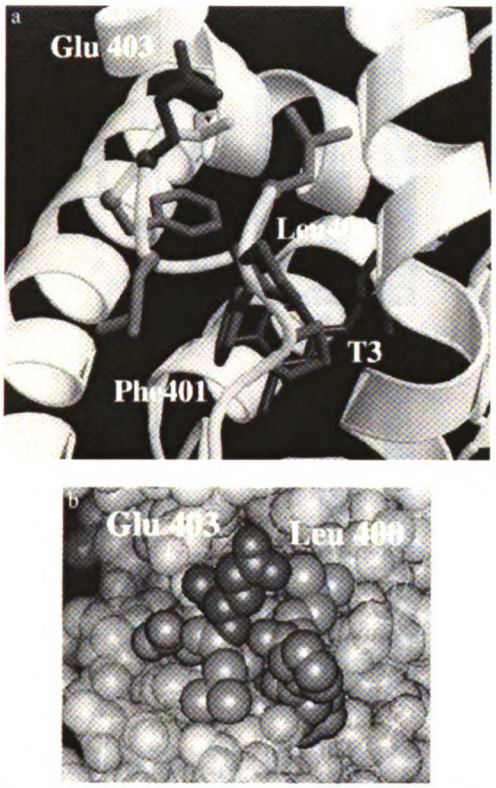

FIG. 3. (*a*) Ribbon drawing of the amphipathic α-helix (399-PLFLEVF-405) from the crystal structure of rat TRα ligand-binding domain, showing the interactions of residues that are important for transactivation. A charged residue, Glu-403 (red), projects toward solvent, whereas a hydrophobic residue, Phe-401 (brown), contacts the ligand T3 (magenta). The residue homologous to Leu-454 in hTRβ, Leu-400 (green), is also solvent accessible. The gray residues (Pro-398, Pro-399) correspond to Pro-452 and Pro-453 in hTRβ. (*b*) Space-filling representation of the rat TRα ligand-binding domain emphasizing the solvent accessibility of Leu-400 and Glu-403. Phe-401 is not visible, since it is buried within the ligand-binding cavity. The view and color scheme are identical to that of Fig. 3*a*.

idue, which is conserved in a number of nuclear receptors (15), in transactivation.

Crystal structures of the ligand-binding domains of hRARγ and rTRα bound to ligand have recently been elucidated (21, 35) and confirm that this C-terminal sequence does indeed form an amphipathic α-helix. In the rTRα ligand-binding domain, some hydrophobic residues in the carboxyl-terminal α-helix (e.g., Phe-401; Fig. 3*a*) are oriented toward the hormone-binding cavity and participate in ligand binding, in keeping with the detrimental effects of mutations at these positions on ligand-binding affinity (16, 18). In contrast, the conserved negatively charged residue (Glu-403) is solvent exposed and is not involved in hormone binding. A similar presentation of Leu-400 (the residue homologous to Leu-454 in TRβ and Leu-543 in mER) on the surface of the receptor raised the possibility that both of these residues might be involved in receptor interaction with heterologous proteins.

A number of proteins that exhibit hormone-dependent interaction with the carboxyl-terminal domains of nuclear receptors have been described. ERAP160 (36) and RIP140 (22, 23) were identified using Far Western blot assays with ER. TIF1 (37), Trip1/Sug1 (38), and SRC-1 (24) were isolated

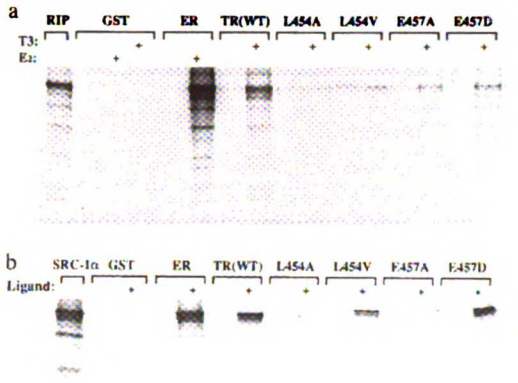

FIG. 4. Interaction of ER, WT TRβ, and transactivation mutants with *in vitro* synthesized RIP140 and SRC-1. ^{35}S-labeled *in vitro* translated RIP140 (*a*) and SRC-1 (*b*) were incubated with equal amounts of glutathione *S*-transferase (GST), GST-ER, or GST-TR fusion proteins in the presence (+) or absence (−) of 1 μM estradiol or T3, as appropriate.

following interaction in a yeast two-hybrid assay system with RAR/RXR, TR, or progesterone receptors, respectively. Most recently, the coactivator CBP has also been shown to interact with nuclear receptors (39). Although the precise role of these proteins remains to be elucidated, their recruitment by liganded receptors probably reflects a realignment of the carboxyl-terminal activation helix in the presence of hormone.

Our data indicate that mutation of either leucine 454 or glutamic acid 457 in the carboxyl-terminal α-helix of TRβ impairs receptor interaction with RIP140 and SRC-1. Furthermore, the most functionally deleterious mutations (L454A, E457A) at these positions are associated with the greatest reduction in RIP140 and SRC-1 binding. More conservative substitutions (L454V, E457D) result in retention of transcriptional activity in some contexts [L454V on a TREpal containing reporter, Fig. 2*d*; a GAL4–E457D fusion on a UASg containing reporter (18)], correlating with retention of some binding to RIP140 or SRC-1. Furthermore, we have shown that coexpressed SRC-1 not only enhances WT receptor activity (Fig. 5*b*), but also augments the function of the L454V mutant, restoring it to WT levels in some TRE contexts. The differing responses to cotransfected SRC-1 exhibited by WT receptor on MAL versus PAL TREs suggests that the transcriptional requirement for this factor may be modulated by TRE configuration. Similarly, the higher transcriptional activity of L454V relative to WT on PAL versus MAL also suggests that the nature of the TRE may influence the ability of the mutant receptor to recruit SRC-1.

Overall, these findings support the notion that hormone-dependent transcriptional activation by TRβ involves receptor interactions with intermediary proteins via critical residues in the C-terminal amphipathic α-helix. Whether other residues from neighboring helices are also involved in such receptor–protein interactions, to constitute an activation surface [as has been suggested for RARγ (35)], remains to be elucidated. Lastly, the association of a transactivation mutation (L454V) with RTH may also have important functional implications for hormone-dependent transcriptional inhibition by TR. Individuals harboring this mutation all exhibit raised serum thyroid hormones with a failure to suppress pituitary TSH secretion, and hormone-dependent negative regulation of a pituitary target gene (the human TSHα promoter) by the L454V mutant receptor is impaired. This suggests that residues in the amphipathic α-helix are also important for hormone-dependent

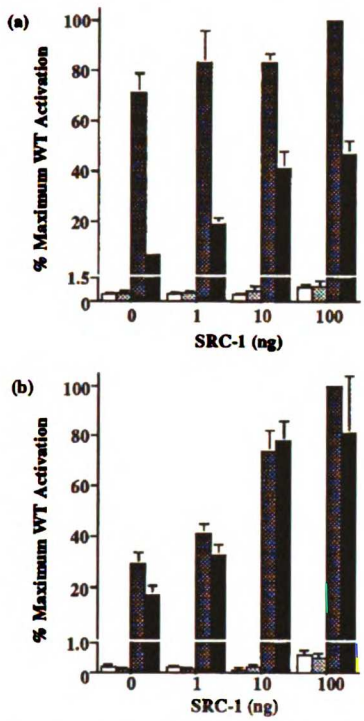

FIG. 5. Effect of coexpressed SRC-1 on the transcriptional activity of WT and L454V mutant receptors. (*a*) 24-well plates of JEG-3 cells were transfected with 500 ng of MALTKLUC, 50 ng of WT or mutant vectors, 100 ng Bos-β-gal and varying amounts (0–100 ng) of SRC-1 expression vector, including empty vector where necessary to keep total vector DNA constant. Following incubation in the absence or presence of 100 nM T3, reporter activity was calculated and expressed as a percentage relative to the maximum corrected luciferase activity of WT receptor with 100 ng of SRC-1. Basal (open) versus stimulated (dark shaded) levels for WT and basal (light shaded) versus stimulated (solid) for L454V mutant receptor are shown. (*b*) JEG-3 cells were transfected with 500 ng of PALTKLUC reporter together with other plasmids as in *a*. Reporter activity with or without 100 nM T3 was calculated as above. Note that the scale of the vertical axes have been interrupted to show both basal and stimulated reporter activity.

transcriptional inhibition by TR, perhaps via interaction with similar accessory factors.

Finally, the properties of the L454V receptor mutant highlight the utility of analyzing naturally occurring TRβ mutations in RTH. Although the majority of natural receptor mutants are dysfunctional as a consequence of altered ligand binding, some are transcriptionally impaired despite normal hormone binding. Providing the mutant receptor also retains dominant negative activity, as in this case, an RTH phenotype should result. Thus, it is likely that other receptor mutants with unexpected properties will be identified in RTH and their analysis may identify further domains involved in transcriptional regulation and protein interaction.

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The following was originally published as "Hormone-Dependent Coactivator Binding to a Hydrophobic Cleft on Nuclear Receptors," by W. Feng, *et. al.* in the June 12, 1998 issue of *Science* (**280**: 1747-1750.) The paper describes an extensive mutagenesis of the TR LBD, which identified a set of five mutations in the human β TR LBD which impaired the interaction of the TR with the p160 coactivator GRIP1. By examining the location of the homologous residues in the structure of the rαTR LBD, the authors infer a site for the interaction with p160 coactivators. The relation of this surface to that observed in the complex between the hTRβ LBD and NR box 2 of the p160 coactivator GRIP1is discussed in Chapter Two of this thesis.

Hormone-Dependent Coactivator Binding to a Hydrophobic Cleft on Nuclear Receptors

Weijun Feng, Ralff C. J. Ribeiro, Richard L. Wagner, Hoa Nguyen, James W. Apriletti, Robert J. Fletterick, John D. Baxter, Peter J. Kushner,* Brian L. West*

The ligand-binding domain of nuclear receptors contains a transcriptional activation function (AF-2) that mediates hormone-dependent binding of coactivator proteins. Scanning surface mutagenesis on the human thyroid hormone receptor was performed to define the site that binds the coactivators, glucocorticoid receptor–interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1). The residues involved encircle a small surface that contains a hydrophobic cleft. Ligand activation of transcription involves formation of this surface by folding the carboxyl-terminal α helix against a scaffold of three other helices. These features may represent general ones for nuclear receptors.

Nuclear receptors stimulate transcription in response to hormone binding by interacting with coactivator proteins in a hormone-dependent manner (*1*). The nature of the nuclear receptor–coactivator interaction site is not known. The site could be a large surface, as is usual with peptide hormone–receptor interactions (*2*), or a small surface with complementarity between the coactivator and nuclear receptor (*3*). Alternatively, the interaction could involve the folding of one protein into the partner (*4*). Coactivator binding is impaired by mutations in the COOH-terminal α helix of the receptor ligand-binding domain (LBD) (*5–7*), but it is not known how this helix contributes to coactivator binding. An autonomous COOH-terminal helix function might be suggested, since the isolated helix confers activation when linked to a heterologous DNA-binding domain (*8*). However, in the liganded thyroid hormone receptor (TR), this helix packs against the LBD and thus the COOH-terminal helix might be part of a larger surface. A conserved Lys outside of the COOH-terminal helix has been implicated in coactivator binding to the estrogen receptor (ER) (*9*), but it is difficult to interpret effects of this single mutation, which might disrupt overall receptor folding (*10*) or affect TR heterodimerization with the retinoid-X receptor (RXR) (*11, 12*).

We mapped the surface of the TR LBD for its interaction with coactivator. We used the TR LBD x-ray crystallographic structure (*13*) to guide placement of 37 mutations in the human (h) TRβ1 LBD surface. Reasoning that bulky, surface-charged residues might disrupt coactivator binding, yet preserve global structure and solubility, we mutated selected residues preferentially to charged residues (Arg, Lys, or Glu) (*14*).

Wild-type (WT) TR and most of the TR mutants liganded to 3,5,3′-triiodo-L-thyronine (T_3) bound equally well to the coactivator glucocorticoid receptor-interacting protein 1 (GRIP1) (Fig. 1A, upper panel) (*15*). In all cases, GRIP1 binding was hormone dependent (*16*). As expected (*5, 6*), mutations $Lys^{454} \rightarrow Arg^{454}$ (L454R) (*17*) and E457K in surface residues of helix 12 abolished GRIP1 binding. Mutations in two residues of helix 3, V284R and K288A, and two residues of helix 5, I302R and K306A, also impaired binding. Five of the mutations with diminished GRIP1 binding (V284R, K288A, I302R, L454R, and E457K) also showed decreased binding to another coactivator, steroid receptor coactivator 1a (SRC-1a) (Fig. 1B) (*18*). Thus, two differ-

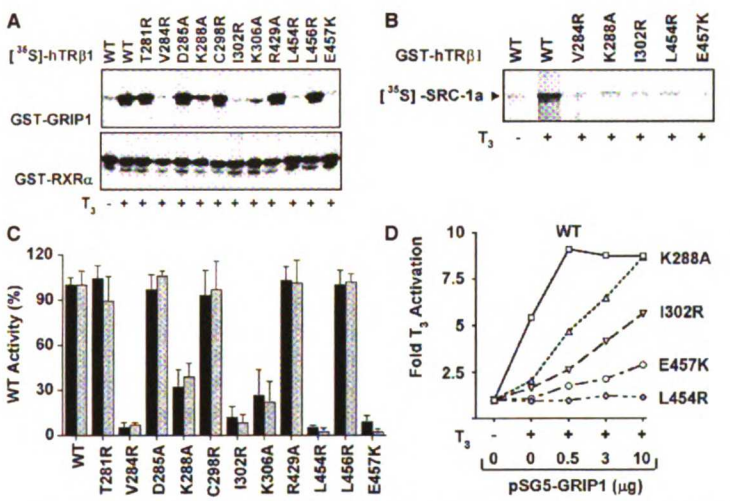

Fig. 1. Binding of GRIP1, SRC-1, and RXRα to hTRβ1 mutants and transcriptional activation by hTRβ1 mutants. (**A**) In vitro binding of ^{35}S-labeled full-length WT and representative mutants of hTRβ1 to GST-GRIP1 (upper panel) and to GST-hRXRα (lower panel). The GST-GRIP1 used included GRIP1 amino acids 721 to 1121; the same results were obtained with a GST-GRIP1 construct that included GRIP1 amino acids 563 to 1121 (*16*). The binding assay was analyzed by autoradiography after separation with 10% SDS–polyacrylamide gel electrophoresis (PAGE) (*25*). The mutations are indicated above the gels. The absence or presence of 1 μM T_3 is indicated below the gels as − or +, respectively. (**B**) In vitro binding of ^{35}S-labeled full-length SRC-1a to WT and mutant GST-hTRβ1. Analysis and display as for Fig. 1A. The same results were obtained when a GST-SRC1 construct including SRC-1a amino acids 381 to 882 was tested for binding of [^{35}S]Met-labeled full-length hTRβ1 WT and mutants (*16*). (**C**) Specific effects of mutations on hTRβ1 transcriptional activation in HeLa cells and correlation with effects on binding to GST-GRIP1. The multiple increases (fold) of T_3 activation (black bar) and of the phosphorimager quantitation of the GST-GRIP1 binding from Fig. 1A (gray bar) are both expressed as percentages of the WT values and graphed for each representative mutant (*25, 26*). Not all 37 mutants are shown. Error bars indicate the SD of three experiments. (**D**) Overexpression of full-length GRIP1 rescued the loss of transcriptional activation by hTRβ1 mutants. Indicated are amounts of the expression vector for full-length GRIP1, pSG5-GRIP1, that were included in the cotransfections, which were otherwise performed as for Fig. 1C (*26*). The WT and representative hTRβ1 mutants are indicated. The data represent the averages of three independent experiments, with SD <10%.

W. Feng, H. Nguyen, J. W. Apriletti, J. D. Baxter, P. J. Kushner, B. L. West, Metabolic Research Unit, Box 0540, University of California San Francisco, San Francisco, CA 94143–0540, USA.
R. C. J. Ribeiro, Department of Pharmaceutical Sciences, University of Brasilia, D.F., Brazil.
R. L. Wagner and R. J. Fletterick, Department of Biochemistry and Biophysics, S1058, University of California San Francisco, San Francisco, CA 94143, USA.

*To whom correspondence should be addressed. E-mail: west@socrates.ucsf.edu (B.L.W.) or kushner@itsa.ucsf.edu (P.J.K.)

ent coactivators recognize the same TR surface residues.

To determine which residues are involved in ligand-mediated transcription activation, we tested the TR mutants in HeLa cells. T_3 increased reporter gene activity fivefold in cells expressing either WT TR or mutated TRs that showed normal GRIP1 binding (representative mutants are shown in Fig. 1C). By contrast, TR mutants with diminished or absent GRIP1 binding (V284R, K288A, I302R, K306A, L454R, and E457K) showed a diminished or absent response to T_3 that correlated with the GRIP1 binding defect. Overexpression of GRIP1 increased activation by the WT TR and rescued activation by TR mutants roughly in proportion to the severity of the defect of GRIP1 binding and activation (Fig. 1D). Thus, the same residues may be required for coactivator binding, functioning of one or more endogenous coactivators in HeLa cells, and responsiveness of TRs to GRIP1; these residues also probably define the TR transcriptional activation function (AF-2).

The effects of the mutations on other receptor functions were also examined. All of the mutants bound radiolabeled thyroid hormone (K_d values, 6 to 234% that for native receptor) (*14*). Occasional lower values were expected because some residues have partially buried side chains; at the high T_3 concentrations used, saturated hormone binding for all mutants was achieved in the assays used. None of the residues that decreased GRIP1 binding affected TR binding to a glutathione S-transferase (GST)–RXR fusion protein (Fig. 1A, lower panel), or to DNA, as determined with three different DNA half-site arrangements testing with or without added RXR (*16*). Some mutations that affect GRIP1 binding occur in a region spanning helices 3 to 5, which has been suggested as important for TR–RXR heterodimerization (*11*, *19*); our results suggest that these AF-2 residues do not contribute to this heterodimerization. Further, mutation of the AF-2 residues does not affect the T_3-dependent inhibition of the Jun and Fos transcription factor activities at an AP-1 site (*5*). These unaffected activities also serve as controls, showing that the amounts of labeled mutant TRs put into the binding reactions and the expression levels of the mutant TRs in the cell culture assays are comparable with those of WT receptors.

The identified TR AF-2 residues lie within portions of the TR sequence that are conserved between nuclear receptors (*10*, *11*, *20*), suggesting that the coactivator-binding surfaces for other nuclear receptors

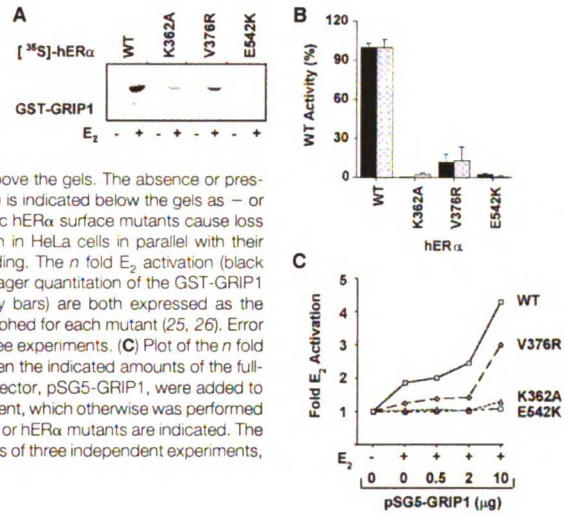

Fig. 2. GRIP1 and binding and transcriptional activation by hERα mutants. (**A**) In vitro binding of WT and mutant ^{35}S-labeled hERα to GST-GRIP1 (GRIP1 amino acids 721 to 1121) (*25*). The mutations are indicated above the gels. The absence or presence of 1 μM estradiol (E_2) is indicated below the gels as − or +, respectively. (**B**) Specific hERα surface mutants cause loss of transcriptional activation in HeLa cells in parallel with their loss of in vitro GRIP1 binding. The *n* fold E_2 activation (black bars) and the phosphorimager quantitation of the GST-GRIP1 binding from Fig. 2A (gray bars) are both expressed as the percentage of WT and graphed for each mutant (*25*, *26*). Error bars indicate the SD of three experiments. (**C**) Plot of the *n* fold E_2 activation observed when the indicated amounts of the full-length GRIP1 expression vector, pSG5-GRIP1, were added to the cotransfection experiment, which otherwise was performed as for Fig. 2B (*26*). The WT or hERα mutants are indicated. The data represent the averages of three independent experiments, with SD <10%.

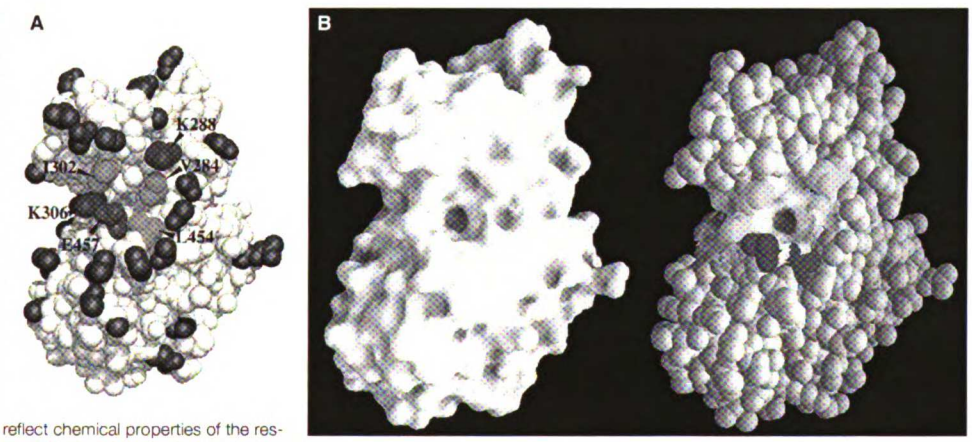

Fig. 3. Scanning surface mutagenesis defines a small cluster of effective mutations that surround a surface cleft containing central hydrophobic residues. (**A**) A space-filling model of the TRα LBD shows the LBD surface locations of mutations made in the full-length hTRβ1. Mutated residues that have no effect on GRIP1 binding or on activation in HeLa cells are shaded dark gray. Mutated residues with diminished GRIP1 and SRC-1a binding and diminished activation in HeLa cells are colored to reflect chemical properties of the residues: Red, blue, and green indicate acidic, basic, and hydrophobic residues, respectively. The main-chain structures of the TRα LBD and TRβ LBDs are the same (*16*). Computer graphic prepared with MidasPlus (*27*). (**B**) The AF-2 surface contains a cleft, one side of which is formed by conformationally hormone-responsive residues. Left, a view of the TR LBD molecular surface, showing the concave surfaces in gray; note the cavity at the center of the figure. Right, a space-filling model of the TR LBD, overlayed with a molecular surface view restricted to a 12-Å radius of the hydrophobic cavity. The hormone-insensitive residues of mutated AF-2 (V284, K288, I302, and K306) are located on one side of the cleft and are colored yellow. The mutated AF-2 residues that likely undergo a conformational change upon hormone binding (L454 and E457) are located on the opposite side of the cleft and are colored red. Computer graphics were prepared with GRASP (*28*).

are similar to that for TR. TR residues K288 and E457 are completely conserved between different nuclear receptors (*10*), and other residues show conservation of residue type—hydrophobic for TR residues V284, I302, and L454, polar or charged for TR residue K306. We created three separate mutations (K362A, V376R, and E542K) in human estrogen receptor-α (hERα), which align to three of the effective positions in the hTRβ1 (K288A, I302R, and E457K). All three mutations diminished GRIP1 binding (Fig. 2A) and abolished transcriptional activation (Fig. 2B); mutant V376R, with 10% residual GRIP1 binding, was rescued partially by overexpression of GRIP1 (Fig. 2C). As a control, the ER mutants demonstrated a normal hormone-dependent ability to activate a vitellogenin-LUC hybrid reporter gene, GL45, which responds to the ER NH_2-terminal activation function (*16*, *21*). Thus, the AF-2 surface residues appear to be similar in different nuclear receptors.

The residues that define AF-2 (Fig. 3A) cluster within a small area with well-defined borders. The surface contains charged and hydrophobic residues at its periphery but only hydrophobic residues at its center. Amino acids that form the surface cleft include residues I280, V284, and K288 from helix 3; I302, L305, and K306 from helix 5; C309 from helix 6; and L454, E457, and V458 from helix 12. This small interaction surface may match a complementary surface on the coactivator with the hydrophobic cleft at the center of the surface driving the coactivator binding reaction. The volume of the cleft, although small (~300 Å^3), can accommodate, for example, a Leu (124 Å^3) or Phe (135 Å^3) residue. A short conserved motif that can mediate recognition of nuclear receptors by coactivators of several types, including GRIP1 and SRC-1 (*22*), contains leucines (LXXLL) that could fit into the target surface hydrophobic cleft. The features of the hydrophobic cleft make it a suitable target for the rational design of pharmaceuticals to block the receptor-coactivator interaction.

The inferred AF-2 surface appears to comprise two parts (Fig. 3B, right). Residues contained in helices 3, 5, and 6 (Fig. 3B, yellow residues) probably form a constitutive part (*10*), since their positions are identical in all nuclear receptor structures reported, including the liganded, activated states of the TR, retinoid-A receptor, and ER, the unliganded RXR, and the inhibitor-liganded ER (*13*, *23*, *24*). By contrast, the residues of helix 12 (Fig. 3B, red residues) are positioned differently in the active and inactive states reported. Thus, the coactivator binding surface is formed in response to an active hormone by positioning helix 12 against a scaffold formed by helices 3 to 6. Because the binding surface is so small, changes in the position of helix 12, such as that induced by the ER antagonist raloxifene (*23*), could impair coactivator binding and thus receptor activation also.

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14. TR mutants were created by ligating double-stranded oligonucleotides to encode the mutant sequence into the pCMX vector that encodes the full-length 461-amino-acid hTRβ1 sequence. Some of the mutations of hTRβ1 in the CMX vector and all three mutations of hERα in the pSG5-ER-HEGO vector [L. Tora *et al.*, *EMBO J.* **8**, 1981 (1989)] were created with the use of Quick Change Site-Directed Mutagenesis Kits (Stratagene). The mutated sequences were verified by DNA sequencing with Sequenase Kits (Stratagene). The T_3 binding affinity constants (K_d) for in vitro–translated WT and mutant TRs were measured by using [^{125}I]T_3 in gel-filtration binding assays as described [J. W. Apriletti, J. D. Baxter, K. H. Lau, B. L. West, *Protein Expr. Purif.* **6**, 363 (1995)]. Both the K_d and standard error were calculated with the Prism computer program (GraphPad Software, San Diego, CA). Mutations are indicated by the single-letter amino acid abbreviations (*17*), with the native residue name being followed by the primary sequence position number and then by the mutated residue name. The affinity of the WT TR is 81 ± 12 pM. The relative affinity was determined by dividing the WT K_d by the K_d for each mutant. The 37 mutants tested, and their relative affinities (%) were: E217R (123%), E227R (109%), K242E (92%), E267R (117%), H271R (123%), T277R (7%), T281R (145%), V284R (105%), D285A (89%), K288A (98%), C294K (94%), E295R (118%), C298A (87%), C298R (141%), E299A (171%), I302A (86%), I302R (99%), K306A (6%), K306E (6%), P384R (164%), A387R (107%), E390R (151%), E393R (146%), L400R (95%), H413R (109%), H416R (153%), M423R (156%), R429A (48%), S437R (170%), L440R (174%), V444R (89%), T448R (234%), E449R (36%), P453E (32%), L454R (26%), L456R (46%), and E457K (71%).
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17. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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25. The WT or mutant pCMV-hTRβ1 vector and the pSG5-GRIP1 and pCMX–SRC-1a vectors were used to produce radiolabeled full-length receptors with the TNT-coupled Reticulocyte Lysate System (Promega) and [^{35}S]Met (DuPont). GST-GRIP1 (amino acids 721 to 1221, from M. Stallcup), GST-GRIP1 (amino acids 563 to 1121, from M. Stallcup), GST–SRC-1a (amino acids 381 to 882), GST-hTRβ1 (full-length, WT or mutants, WT from C. Costa), and the GST-hRXRα (full-length, from C. Costa) fusion proteins were produced in *Escherichia coli* strain HB101 according to the manufacturer's protocol (Pharmacia Biotech). The binding experiments were performed by mixing glutathione beads containing 10 μg of GST fusion proteins (Coomassie Plus Protein Assay Reagent, Pierce) with 1 to 2 μl of the ^{35}S-labeled WT or mutant hTRβ1 (25 fmol, 4000 cpm of receptor), or 1 to 2 μl of coactivators, in 150 μl of binding buffer (20 mM HEPES, 150 mM KCl, 25 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors) containing bovine serum albumin (2 μg/ml) for 1.5 hours in the presence or absence of 1 μM T_3. After the beads were washed three times with 1 ml of binding buffer, the bound proteins were separated with use of 10% SDS–PAGE and visualized by autoradiography. Binding was quantitated by phosphorimaging with ImageQuant (Molecular Dynamics).
26. HeLa cell transfection and assay conditions are described [P. Webb, G. N. Lopez, R. M. Uht, P. J. Kushner, *Mol. Endocrinol.* **9**, 443 (1995)]. For TR assays, we used 5 μg of the reporter p(DR-4)$_2$–TK-LUC consisting of two copies of the DR-4 element [a direct repeat of the consensus TR response element (TRE) spaced by 4 bp] placed upstream of a minimal (−32/+45) thymidine kinase gene promoter linked to luciferase coding sequences. A reporter containing palindromic TREs gave the same results (*16*). We used 2 μg of the TR expression vector, pCMX-TR (WT or mutant), and 0.5 μg of the control vector, pJ3LacZ, which contains the SV40 promoter linked to the β-galactosidase (β-Gal) gene. For the hERα assays, we used 5 μg of estrogen-responsive reporter plasmid encoding chloramphenicol acetyltransferase (CAT), pERE-ColITATA [Y. Sadovsky *et al.*, *Mol. Cell. Biol.* **15**, 1554 (1995)], 0.5 μg of expression vector encoding full-length hERα, pSG5-ER HEGO (WT or mutants), and 2 μg of pJ3LacZ. For the experiments of Figs. 1D and 2C, 0.5 μg of a full-length GRIP1 expression vector, pSG5-GRIP1, was also included in the transfection. Transfected cells were treated with or without 1 μM T_3 or E_2, as indicated. After cell culture for 24 hours, the LUC or CAT activities were assayed; β-Gal activities were also assayed to correct for differences in transfection efficiencies.
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29. We thank M. Stallcup and C. Costa for providing the GST-GRIP1, GST-RXRα, and GST-TRβ expression vectors; P. Webb and M. Tsai for providing the pSG5-GRIP1 and pCMX–SRC-1a expression vectors; K. Yamamoto, P. Webb, M. G. Rosenfeld, and T. Scanlan for suggestions; and T. Chen and K. Nakamura for technical assistance. W.F. is a recipient of a postdoctoral fellowship from the National Institutes of Health (DK09516). Supported by NIH grants to J.D.B. (DK51281) and to the Computer Graphics Laboratory at the University of California, San Francisco (P41-RR01081).

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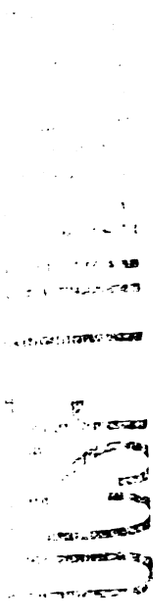


Appendix Two

Protocols

Crystallization of rat α_1TR LBD

Two crystallization conditions were identified for the rat α isoform of the thyroid hormone receptor ligand binding domain (Met^{122} to Val^{410}), purified as described in Apriletti, J. W., *et al. Protein Expr. Purif.* **6**(3): 363-370 (1995).

Form I

monoclinic crystals of space group C2 (a=117.16 Å, b=80.52 Å, c=63.21 Å, β=120.58)

Crystals are grown using hanging drop vapor diffusion at ambient temperature (17°C to 22°C), from drop containing a 2:1 mixture of approximately 10 mg/ml protein, in 20mM Hepes pH 7.4 and 3 mM DTT, and a reservoir solution of 15% 2-methyl-2,4-pentanediol [MPD], 0.2 M ammonium acetate [NH_4OAc],buffered by 0.1 M sodium cacodylate at pH 6.7. The crystals grow in the presence of a fluffy, white precipitate. Isomorphous crystals of the LBD bound to either 3,5,3'-triiodothyronine (T_3), 3'-isopropyl-3,5-dibromothyronine ($IpBr_2$), or 3'-isopropyl-3,5-dimethylthyronine (Dimit) can be prepared from these conditions, though the optimal protein concentration differs between ligands.

The crystals may be frozen in a nitrogen stream directly from the mother liquor; the MPD concentration must be increased if the crystals are frozen liquid N_2. If the crystal does not freeze rapidly (for example, frozen with an excess of mother liquor in a loop of dimensions greater than those of the crystal), the unit cell shrinks along **c** (from 63Å to 57Å).

Data collection for cocrystals of the $rTR\alpha_1$ LBD with Dimit, T_3, and $IpBr_2$ is described in Chapter One.

Form II

hexagonal crystals of space group $P6_522$ (a=b=108.64 Å, c=133.72 Å)

Crystals are grown using hanging drop vapor diffusion at 17°C, from a drop containing a 1:1 mixture of approximately 10 mg/ml protein in 20mM Hepes pH 7.4 and 3 mM DTT, and a reservoir solution of 0.45 M ammonium phosphate [$(NH_4)H_2PO_4$], 0.2 M ammonium sulfate [$(NH_4)_2SO_4$] buffered at pH 7.0 with potassium sulfate [0.1 M KH_2PO_4].

The crystals may be frozen in a nitrogen stream using a cryoprotectant composed of 30% glycerol, 0.5 M $(NH_4)H_2PO_4$, 0.2 M $(NH_4)_2SO_4$, and 0.1 M KH_2PO_4 pH 7.0. A complete data set to 2.4 Å (T_3) was measured at -170°C at SSRL (λ = 1.08Å) on a MAR detector.

Crystallization of human β TR LBD

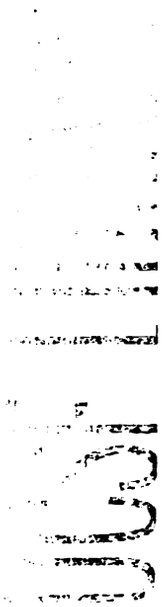
Two crystallization conditions were identified for the human β isoform of the thyroid hormone receptor ligand binding domain (His_6 Glu^{202} to Asp^{461}), purified as described in Shiau, A., unpublished.

Form I

trigonal crystals of space group P3$_1$21

Crystals are grown using hanging drop vapor diffusion at 4°C from 1.0 M sodium acetate [NaH_3OAc] buffered by 100 mM sodium cacodylate [NaCac] pH 7.2.
The N-terminal His-tag was not removed prior to crystallization.

The crystals may be frozen in liquid N_2 using a cryoprotectant composed of 30% glycerol, 1.4 M NaH_3OAc, and 100 mM NaCac pH 7.2; not every crystal can be frozen. A complete data set to 2.4 Å (Triac) was measured at -170°C at SSRL (λ = 1.08Å) on a MAR detector; data extending to 3.2 Å for T_3 were measured at -170°C at UCSF using an *R*-axis rotating Cu anode source ((λ = 1.54Å) and an *R*-axis-II IP area detector.



Cocrystallization with GRIP1 NR-box 2 peptide

trigonal crystals of space group $p3_121$ (a=95.2 Å, c=137.6 Å)

The complex was prepared by mixing 9 mg/ml TRβ LBD in 20 mM HEPES pH 7.4, with 14 mM peptide in 0.4 mM ammonium acetate pH 4.72, at a 1:2 ratio, and kept on ice for 1 hour. Crystals are obtained after 2 days by hanging drop vapor diffusion at 4°C from a drop containing 1.5 µl of TRβ LBD:NR-box 2 peptide complex, and 0.5 µl 15% polyethylene glycol 4000 MW (PEG 4K; Hampton), 200 mM sodium citrate [NaCit] pH 4.9, suspended above a reservoir containing 10% PEG 4K, 100 mM ammonium acetate [NH_4OAc], and 50 mM sodium citrate (pH 5.6). An hour after preparing the drops, 0.2µl of 10^{-3} to 10^{-5} dilutions of microcrystals in reservoir buffer are introduced to promote nucleation.

Crystals were soaked overnight in a drop composed as described above plus 10% sucrose; then transferred serially, 5 min each, to drops containing 10% PEG4K, 100 mM ammonium acetate, and 50 mM sodium citrate (pH 5.6), 10% sucrose, excess NR-box 2 peptide, and increasing concentrations of xylitol (2% to 13% by 2% steps). Crystals were then flash frozen in liquid nitrogen). A complete data set to 3.6 Å was measured at -170°C at UCSF using an *R*-axis rotating Cu anode source (λ = 1.54Å) and an *R*-axis-II IP area detector.

Appendix Three

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GRADUATE GROUP IN BIOPHYSICS
tel: (415)-476-5051
fax: (415)-476-1902
EMAIL: WAGNER@MSG.UCSF.EDU

UNIVERSITY OF CALIFORNIA
BOX 0448
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tel: (415)-476-5051
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EMAIL: WAGNER@MSG.UCSF.EDU

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fax: (415)-476-1902
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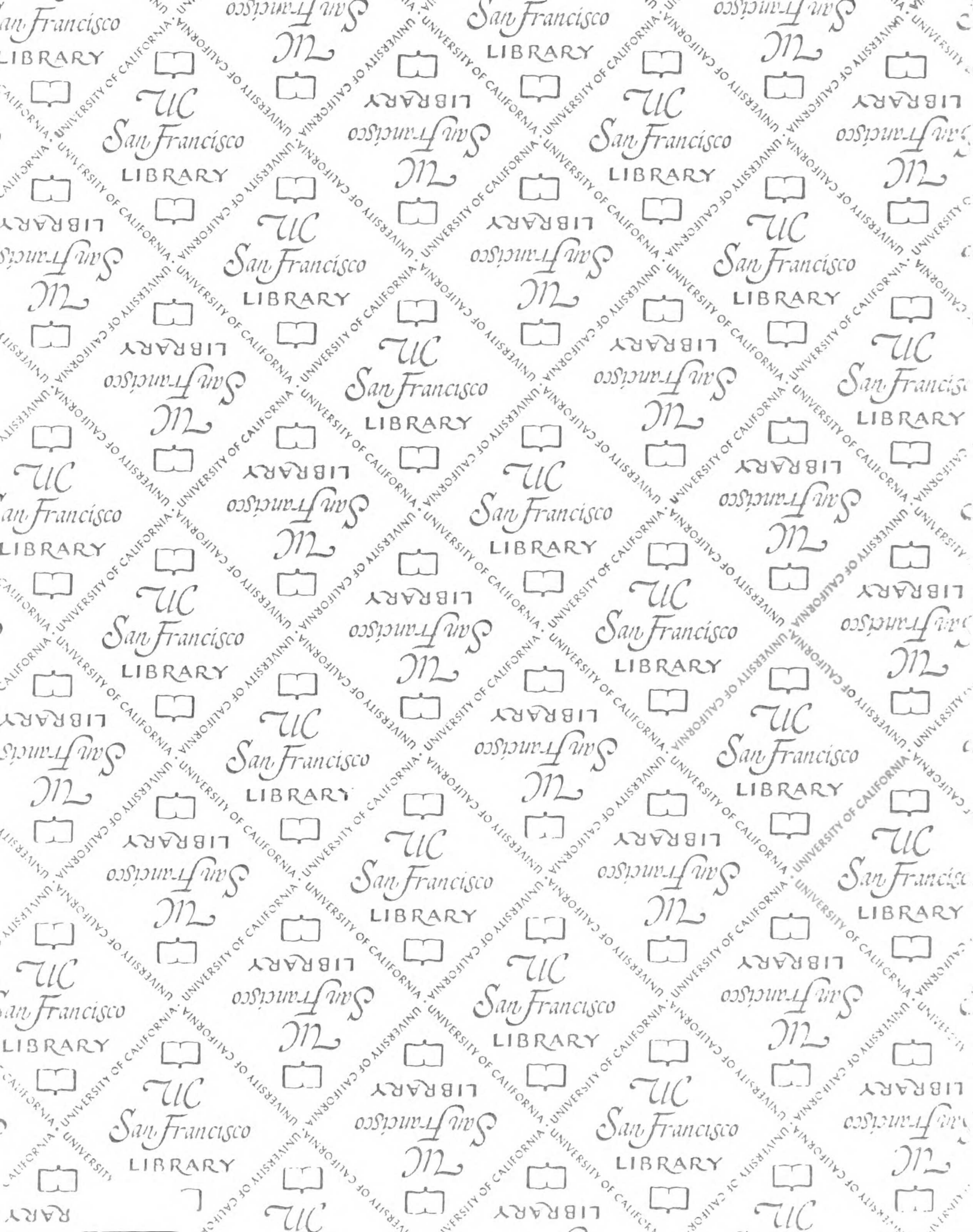
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