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Structural and Biochemical Investigations of the Thyroid Hormone Receptor and v-erbA Oncoprotein

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Structural and Biochemical Investigations of the Thyroid Hormone Receptor and v-erbA Oncoprotein

by

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DISSERTATION

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This work is dedicated to my parents,

who fostered my curiosity from my earliest days,

and to Laura,

who supported me every step of the way.

Structural and Biochemical Investigations of the Thyroid Hormone Receptor and v-erbA Oncoprotein

Eric John Slivka

Abstract

The thyroid hormone receptor is a ligand-dependent transcription factor crucial for the regulation of a wide variety of processes involved in metabolism, differentiation, and development. While much has been learned about the structure of the thyroid hormone receptor and the molecular determinants of hormone binding, a complete understanding of these determinants has not yet been obtained.

In order to better understand the molecular determinants of hormone binding in the thyroid hormone receptor, v-erbA, a naturally occurring form of the receptor found in a retrovirus, was used as a platform for mutational studies. The use of v-erbA, which is severely compromised in its ability to bind thyroid hormone and activate transcription, provided a convenient and natural template upon which targeted mutations could be made.

Mutational analysis on ligand binding domain constructs determined that the addition of a missing helix (Helix 12) near the C-terminus of v-erbA was insufficient to restore ligand binding ability to the receptor, but an additional

single point mutation, S395F, near the same location was capable of restoring hormone affinity to within ten-fold of that of wild-type thyroid hormone receptor. Protein stability assays based on protease degradation and thermal denaturation also demonstrated this constructs improved stability over other v-erbA constructs.

Transcriptional data obtained using full-length constructs in transient transfection assays demonstrated that the S395F mutation significantly enhanced the ability of v-erbA to activate transcription in the presence of hormone. While both v-erbA and v-erbA + Helix 12 constructs not only failed to activate transcription in the presence of hormone but maintained repression seen in the absence of hormone, the v-erbA + Helix 12 S395F mutant activated transcription even beyond the level seen with wild-type thryroid hormone receptor.

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An Introduction to Thyroid Hormone Receptor and v-erbA

Nuclear Receptors

Nuclear receptors are a superfamily of ligand-dependent transcription factors implicated in the regulation of a wide variety of processes involved in metabolism, differentiation, and development. Historically, members of the nuclear receptor family have been identified based on sequence and domain homology (Evans, 1988; Mangelsdorf et al., 1995; Tsai & O'Malley, 1994).

The nuclear receptor superfamily is typically divided into three classes. Class I nuclear receptors consist of the steroid receptors and include the androgen receptor, the estrogen receptor, glucocorticoid receptor, mineralocorticoid receptor, and the progesterone receptor.

Nuclear receptors typically consist of three primary domains and several auxiliary domains. Most receptors possess an N-terminal domain (NTD), also known as the A/B domain or activation function 1 (AF-1) domain. While the NTD varies greatly in size among nuclear receptors and is entirely absent in some, the domain is generally associated with ligand-independent transcriptional activation (Jenster, van der Korput, Trapman, & Brinkmann, 1995; Langlois et al., 1997; Wilkinson & Towle, 1997). No high-resolution structure of a nuclear receptor NTD has been determined, presumably due a high degree of disorder within the domain and the requirement of interactions with binding partners to stabilize the domain (McEwan, Lavery, Fischer, & Watt, 2007).

The nuclear receptor DNA-binding domain (DBD), also referred to as the C domain, contains two zinc finger motifs that are responsible for recognition of specific DNA response elements (Freedman, 1992). These response elements (RE) are composed of six-base half-sites of variable spacing that can be bound by nuclear receptor monomers, homodimers, and/or heterodimers (Glass, 1994). The arrangement of these half-sites can be classified into three categories: 1) Symmetric sites, typically with a spacing of three bases between the half-sites; 2) Direct repeats, in which spacing can vary between one and five bases; and 3) Monomeric sites, which generally contain specific 5' flanking sequences used to target certain monomeric nuclear receptors (Khorasanizadeha & Rastinejad, 2001). The DNA-binding domain also participates in dimerization of nuclear receptors that stabilizes the interactions of the receptor dimer with the response elements (Perlmann, Rangarajan, Umesono, & Evans, 1993; Zechel, Shen, Chambon, & Gronemeyer, 1994).

The hinge region, also known as the D domain, links the DBD to the ligand-binding domain and provides flexibility for rotation of the two domains with respect to one another. The hinge is also thought to be involved in interactions between the nuclear receptor and both corepressors (Chen & Evans, 1995; Hörlein et al., 1995; Kurokawa et al., 1995) and coactivators (Chakravarti et al., 1996; Halachmi et al., 1994; Kamei et al., 1996; Oñate, Tsai, Tsai, & O'Malley, 1995)

The ligand-binding domain (LBD) generally consists of twelve helices forming a hydrophobic pocket for which almost entirely encloses the ligand (Figure 1-1). In the case of nuclear receptors that do not bind ligand, the pocket is largely collapsed and filled with the sidechains of amino residues lining the pocket. In addition to ligand binding, the LBD is also involved in transcriptional activation, nuclear localization, and dimerization (Holloway, Glass, Adler, Nelson, & Rosenfeld, 1990; Picard, Salser, & Yamamoto, 1988).

The various functions of the LBD map to various locations throughout the domain. Ligand-binding functions extend throughout the domain, as the hydrophobic binding pocket is lined by residues from a number of different helices in the domain (Evans, 1988; Muñoz et al., 1988). The LBD's nuclear localization sequence, a highly basic region, is found near the N-terminus of the LBD (Boucher, Koning, & Privalsky, 1988), while dimerization and other protein-protein interactions are mediated through a small region primarily consisting of portions of two helices within the ligand-binding region (Forman & Samuels, 1990).

The C-terminal domain, or F domain, is variable in length among the nuclear receptors, and completely absent in some. The domain's function is currently unknown (Robinson-Rechavi, Escriva Garcia, & Laudet, 2003).

Thyroid Hormone and its Receptor: Early Discoveries

The most prevalent of the thyroid hormones in the blood stream, known as 3,5,3',5'-tetraiodo-L-thyronine or T_4, was discovered in 1915 (Kendall, 1915), but it was not until the early 1950s that the more potent hormone, 3,5,3'-triiodo-L-thyronine or T_3, was isolated, characterized and synthesized (Gross & Pitt-Rivers, 1952) (Figure 1-2). The cellular site of action of thyroid hormone, however, remained unknown until the discovery a decade a later of its association with DNA transcription (Tata, 1963; Tata & Widnell, 1966). By the early 1970s, high-affinity receptors for thyroid hormone had been discovered (Oppenheimer, Koerner, Schwartz, & Surks, 1972; Samuels & Tsai, 1973).

Avian Erythroblastosis Virus

The history of avian erythroblastosis virus (AEV) dates to the mid-1930s, when Rothe-Meyer and Engelbreth-Holm reported the discovery and isolation of several strains of an infectious agent capable of inducing leukemia of red blood cells in birds (Engelbreth-Holm & Rothe-Meyer, 1935). Rothe-Meyer and Engelbreth-Holme were able to isolate a stable strain, which they termed AEV-ES4/R, and demonstrated that the agent was in fact a retrovirus (Engelbreth-Holm & Rothe-Meyer, 1935). Following these initial discoveries, AEV research lay dormant for several decades. Eventually, AEV research came back into favor

(Ishizaki & Shimizu, 1970), and it was discovered that not only could AEV cause erythroleukemia, but it could also cause slow-growing sarcomas of virally-transformed fibroblasts in infected animals (Graf, Royer-Pokora, Schubert, & Beug, 1976).

By the early 1980s, the proteins encoded by AEV had been identified, with the two primary loci being named v-erbA and v-erbB (Anderson, Hayward, Neel, & Hanafusa, 1980; Vennström & Bishop, 1982). The v-erbB gene, which was later discovered to be a mutated version of the epidermal growth factor (EGF) receptor (Ullrich et al., 1984), was determined to be the more potent of the two oncogenic genes, as it demonstrated the ability to induce both leukemia and sarcomas even in the absence of the v-erbA gene (Frykberg et al., 1983). The constitutively active v-erbB protein causes cellular transformation by mimicking the signals generated by epidermal growth factor (Privalsky, 1992).

The v-erbA protein, on the other hand, is generally not capable of inducing cellular transformation on its own (Frykberg et al., 1983). Instead, the protein prevents cells transformed by v-erbB from differentiating into mature erythrocytes (Frykberg et al., 1983). This maintains the erythrocytes in an immature and highly proliferative state that is the hallmark of leukemias.

The Cellular Counterpart of v-erbA is a Thyroid Hormone Receptor

The research lines of thyroid hormone receptor and AEV first crossed in 1985 when cloning of the estrogen receptor and glucocorticoid receptor revealed close similarities to v-erbA and the cellular version, c-erbA, from which it was derived (Green et al., 1986; Hollenberg et al., 1985; Weinberger, Hollenberg,

Rosenfeld, & Evans, 1985). Not long after, the c-erbA gene was in fact determined to encode for a thyroid hormone receptor (Sap et al., 1986; Weinberger et al., 1986).

Thyroid Hormone Receptor Gene Products

Thyroid hormone receptors are actually encoded by two separate gene loci, denoted as TRα (or c-erbA α) and TRβ (or c-erbA β). Each of these gene products is also expressed in two alternative splice variants, leading to a total of four TR-like proteins: TRα1, TRα2, TRβ1, and TRβ2.

Of the two TRα subtypes, only TRα1 is capable of binding thyroid hormone. The TRα2 subtype does not bind hormone, and also differs from TRα1 in its ability both bind DNA and heterodimerize with its partner nuclear receptor, the retinoid X receptor (RXR) (Izumo & Mahdavi, 1988; Katz, Berrodin, & Lazar, 1992). TRα2 is believed to function primarily as a transcriptional repressor, modulating normal TR function in the cell (Tagami, Kopp, Johnson, Arseven, & Jameson, 1998). Both TRβ isoforms are able to bind hormone and activate transcription, although they exhibit varying dimerization and transcriptional control. (Ng, Forrest, Haugen, Wood, & Curran, 1995).

The four isoforms of thyroid hormone receptor are subject to differential regulation of their expression patterns. TRα1 is widely expressed in a variety of tissues from early embryogenesis, and TRα2, although frequently coexpressed with TRα1, is seen at its highest levels in the brain and testes (Benbrook & Pfahl, 1987; Mitsuhashi & Nikodem, 1989). TRβ1 is expressed in many of the same

tissues as TRα1, although it's expression profile is shifted to late embryogenesis (Forrest, Sjoberg, & Vennstrom, 1990). Expression of TRβ2 is generally limited to the pituitary gland, inner ear, and developing neuronal cells (Bradley, Towle, & Young, 1992; Hodin et al., 1989; Wood, Ocran, Gordon, & Ridgway, 1991).

Comparison of Chicken TRα1 and v-erbA LBDs

Unlike c-erbA, v-erbA will not measurably bind T_3 at reasonable hormone concentrations (Muñoz et al., 1988). This, in conjunction with slightly altered DNA-binding affinity, is thought to be the mechanism by which v-erbA interferes with normal TR function. v-erbA is able to compete very effectively with TR for the TRE binding sites, and does not respond to the presence of hormone, resulting in the observed constitutive repression of the target genes. An analysis of the chicken TRα1 and v-erbA LBDs reveals a few significant alterations in the viral form of the gene. Most importantly, a nine amino acid residue section of the LBD, corresponding to helix 12, has been deleted. It would be reasonable to assume that this deletion is responsible for the lack of hormone binding by v-erbA, and this may well be the case. However, data has shown that a naturally occurring human mutant of TRβ, truncated at F395, still binds T_3 with a K_d of 100 nM, roughly 1000-fold worse than wild-type (Marimuthu et al., 2002). Thus, the deletion of helix 12 in v-erbA is likely not the sole factor contributing to the loss of ligand binding ability observed. Notably, unlike the human truncation at F395, the three C-terminal residues are present in v-erbA, and their interactions with the binding pocket in this "new" location are not known. In addition to the helix 12 deletion, the v-erbA LBD also contains nine additional point mutations.

Most of the mutations are not in the vicinity of the binding pocket, and are in fact surface residues. Also of interest is the observation that three of the nine mutations are prolines that have been mutated to either leucine or serine. Whether increased flexibility generated by these mutations contributes to loss of ligand binding or if a more specific structural change is the cause is unknown (Figures 1-3 and 1-4).

Thyroid Hormone Mechanism of Action

Thyroid hormone mediates the expression of a number of target genes, thus regulating numerous physiological processes. Thyroid hormone is generally slow acting (on the order of hours or days), and affects processes ranging from basal metabolic rate to fetal development to cardiac function.

After synthesis in the thyroid gland, thyroid hormone is secreted into the bloodstream, where it is dispersed through the body. While small quantities of thyroid hormone, estimated at 0.4% of T_3 and 0.04% of T_4, do freely circulate in the bloodstream, T_3 and T_4 are typically bound to carrier proteins, including thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA), TBG contains a single high-affinity binding site for both T_3 and T_4 and is responsible for transporting approximately 70% of thyroid hormone in the bloodstream. Most of the remaining 30% of circulating thyroid hormone is carried by lower-affinity proteins, which serve as the primary source of free hormone in tissues (Cooper, Greenspan, & Ladenson,). Upon arrival at the target tissues the thyroid hormones are actively transported in to cells by the monocarboxylate transporter 8 (MCT8) (Friesema et al., 2003).

Much has been learned in recent years regarding auxiliary proteins involved in repression and activation of TR-responsive genes. The two major corepressor proteins that interact with TR in the absence of hormone are NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) (Chen & Evans, 1995; Hörlein et al., 1995) . Two helical receptor interaction domains (RIDs) containing an isoleucine motif (I/LXXII) have been described in both of these 270 kDa proteins, and a third RID has recently been found in NCoR (Webb et al., 2000). In addition, both NCoR and SMRT contain three repression domains that serve to recruit additional proteins involved in histone deacetylation and repression of the basal transcriptional machinery.

Upon ligand binding, corepressors dissociate from TR and coactivators are recruited. A number of coactivator proteins have been identified, including SRC-1 (steroid receptor coactivator 1), SRC-2/TIF2 (transcriptional intermediary factor 2)/GRIP1 (glucocorticoid receptor interacting protein 1), SRC3/p300/ACTR, DRIPs (vitamin D receptor interacting proteins)/TRAPs (thyroid hormone receptor accessory proteins), and CBP (CREB binding protein) (Harvey & Williams, 2002; Yen, 2001). The exact role of all of these coactivator is not presently known, although there do appear to be at least two major

complexes that play significant role in transcriptional activation, the SRC complex and the DRIP/TRAP complex.

These coactivators contain RIDs with LXXLL motifs shown to be important for nuclear receptor – coactivator binding (Heery, Kalkhoven, Hoare, & Parker, 1997). Coactivators also contain multiple regions that appear to interact with various other proteins, including CBP/p300 (which may serve in the integration of multiple signaling inputs), histone acetyltransferases, and other general transcription factors (Korzus et al., 1998). While significant progress in identifying components of the TR complexes has been made, much remains to be determined in understanding their interactions.

In addition to transcriptional effects, thyroid hormones have also been shown to elicit rapid, non-genomic effects on much faster timescales than those possible through thyroid hormone receptors (Falkenstein, Tillmann, Christ, Feuring, & Wehling, 2000). For example, 3-iodothyronamine (T_1AM), a naturally-occurring derivative of thyroid hormone, has been demonstrated to be a potent agonist of the G-protein coupled receptor TAR1 and is capable of producing hypothermia and bradycardia and reducing cardiac output within minutes of application (Scanlan et al., 2004).

Thyroid Hormone Receptor Knockouts and Knockins

Recent studies on targeted gene inactivation ("knockouts") of thyroid hormone receptors isoforms, as well as replacement of native TR genes with mutant versions ("knockins") have provided additional insight into the mechanisms of thyroid hormone action (Flamant & Samarut, 2003; Forrest &

Vennstrom, 2000). Disruption of TR genes by targeted mutagenesis is complicated by the fact that the TRα gene encodes not only the alternatively spliced TRα1 and TRα2 variants, but also Rev-erbA, which is generated from the opposite strand of TRα (Yen, 2001). Consequently, several TRα knockout mouse lines have been created that display different phenotypes (Fraichard et al., 1997; Wikstrom et al., 1998), likely due to differences in the gene locus used for recombination to generate the knockout mice.

TRα -/- mice lacking both TRα1 and TRα2 exhibit a severe phenotype of hypothyroidism, growth retardation intestinal malformation, and early death shortly after weaning, although treatment with injections of T_3 prevent the early death of the mouse pups (Wikstrom et al., 1998). TRα1 -/- mice lacking only the primary TRα1 gene exhibit a milder phenotype of decreased body temperature and prolonged QT intervals on electrocardiograms (Fraichard et al., 1997). Disruption of the TRα1 causes decreased heart rate and prolonged QRS and QT durations, effects that persist after addition of hormone. No changes are seen in the levels of known thyroid hormone-responsive cardiac genes, such as the Na+-K+ ATPase, and β-adrenergic receptors. TRα1-deficient mice also have a C reduction in body temperature of 0.5^{o} that is independent of thyroid hormone levels (Fraichard et al., 1997).

Additional work has used intronic transcriptional start sites to generate short TRα isoforms which demonstrate dominant negative activity on thyroid hormone receptor function (Chassande et al., 1997) It is possible that these short TR⟨ isoforms are responsible for the increased severity of phenotype seen in TRα

-/- mice, as TR⟨ knockout mice which did not express any isoforms of TRα1 or TRα2 exhibited a milder phenotype than TRα -/- mice still expressing short TRα isoforms (Flamant & Samarut, 2003; Forrest & Vennstrom, 2000).

Disruption of the TRβ gene locus yields mice deficient in both TRβ1 and TRβ2 (Flamant & Samarut, 2003; Forrest & Vennstrom, 2000). These mice exhibit elevated levels of circulating thyroid-stimulating hormone (TSH) and T_4, hearing defect, and thyroid hyperplasia (Forrest et al., 1996; Weiss et al., 1997).

The relatively mild phenotypes of the TRα1 and TRβ knockout mice suggest redundant roles for two isoforms in the transcriptional regulation of many target genes. In microarray studies designed to address this issue, TRα and TRβ knockout mice exhibit regulation profiles in both the absence and presence of T_3 (Yen et al., 2003). Interestingly, double knockout mice (TRα1-/- TRβ -/-) are viable, although they exhibit elevated T_4, T_3, and TSH levels as well as large goiters and decreased growth, heart rate, fertility, and bone density and development.

Knockin mouse models have also been developed to study the mechanism of thyroid hormone action. In one such line, a mutant TRβ from a human patient with resistance to thyroid hormone (RTH) was substituted for the mouse's own TRβ gene (Kaneshige et al., 2000). Heterozygous mice exhibit a phenotype similar to RTH patients, with elevated circulating T_4 and TSH levels, mild goiter, hypercholesterolemia, impaired weight gain, and abnormal bone development, while homozygous mice had markedly elevated serum T4 and TSH, and a much more severe phenotype than heterozygous mice.

Additional studies on knockins of mutant versions of TRβ have revealed a effects on the brain, with abnormal cerebellar development and function, and learning deficits (Hashimoto et al., 2001).

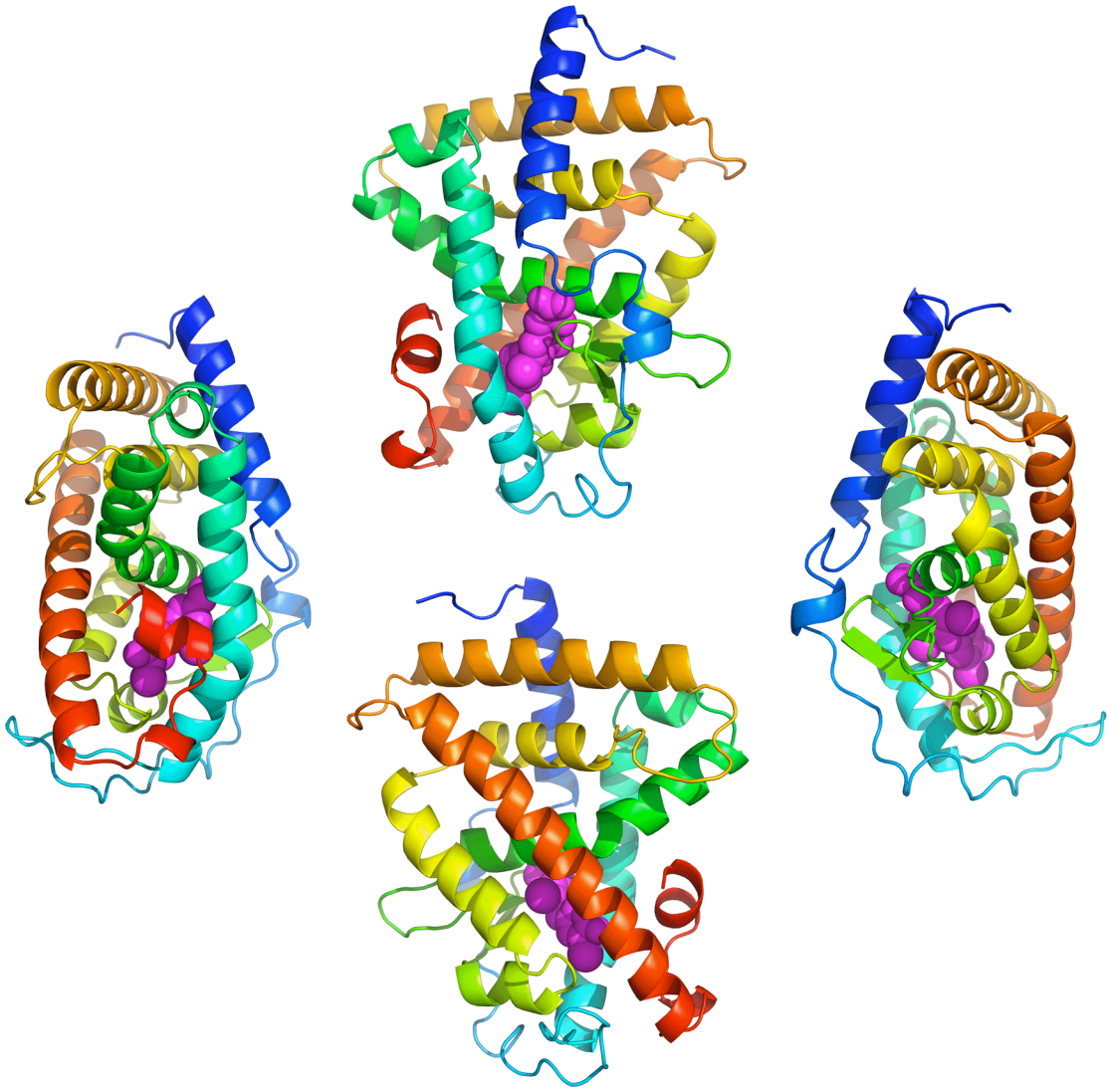

Figure 1-1: Crystal structure of rat thyroid hormone receptor α ligand binding domain bound to T_3.

The crystal structure of the rat thyroid hormone receptor a ligand binding domain bound to T_3 was the first determined high-resolution structure of a nuclear receptor ligand binding domain (Wagner et al., 1995). Shown are four views, the canonical front and side views, as well as two side views.

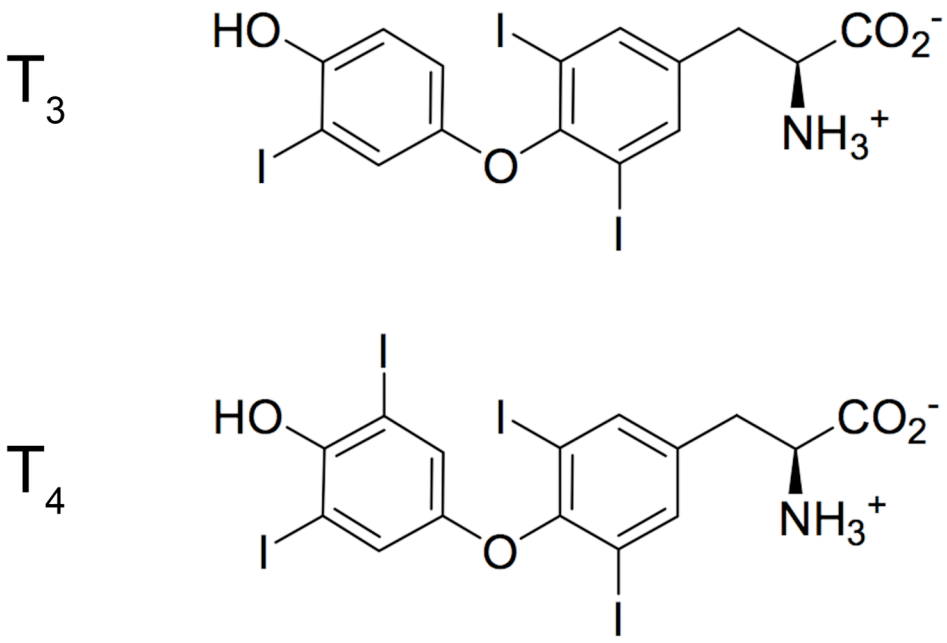

Figure 1-2: Structures of thyroid hormones 3,5,3'-triiodo-L-thyronine (T_3) and 3,5,3',5'-tetraiodo-L-thyronine (T_4).

T_3, the more potent thyroid hormone, is formed by the action of deiodinases on the more prevalent T_4, which is itself synthesized in the thyroid using tyrosine amino acid molecules as the basic building block.

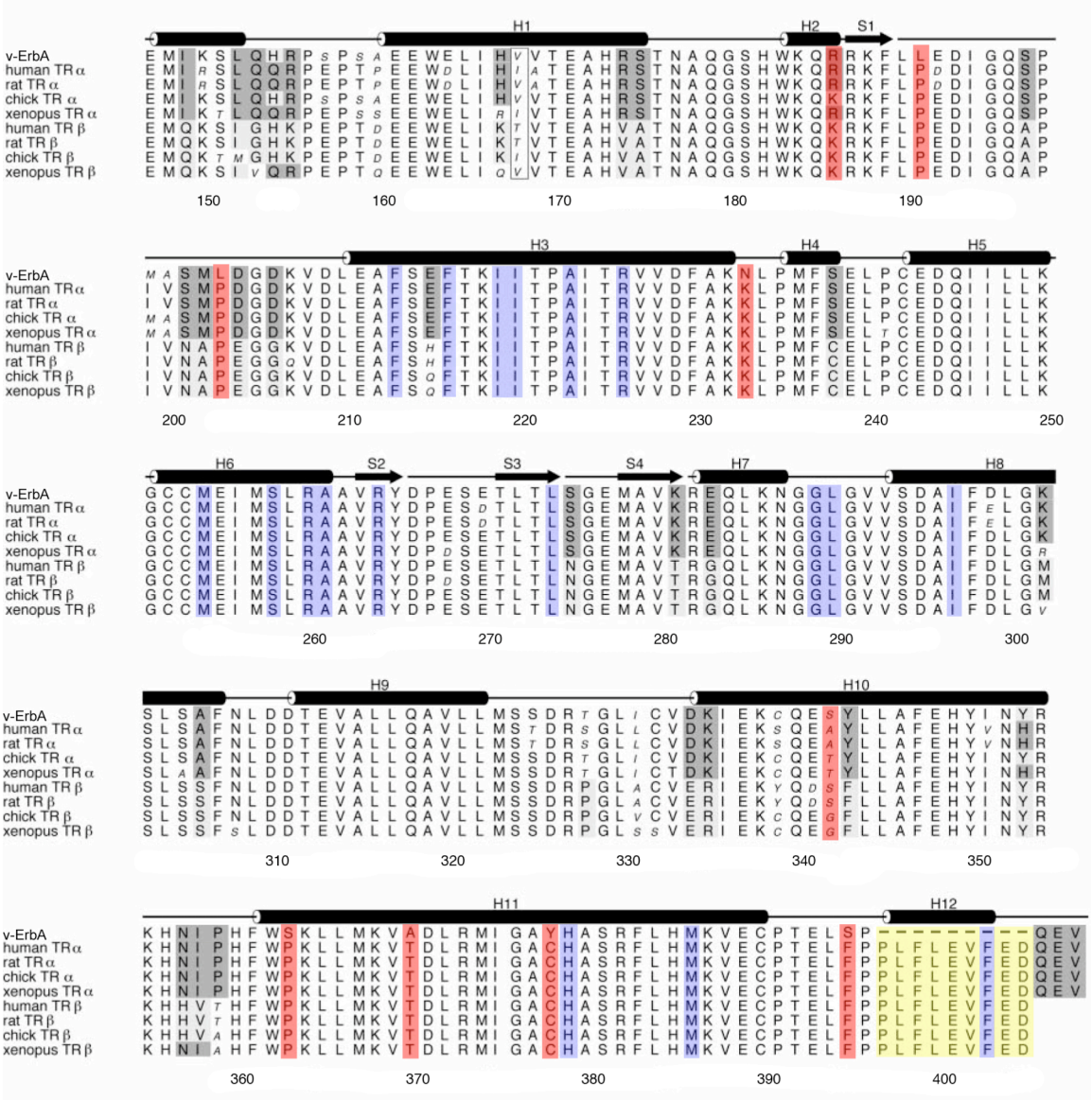

Figure 1-3: Sequence alignment of v-erbA ligand binding domain with thyroid hormone α and β ligand binding domains from human, rat, chicken, and frog.

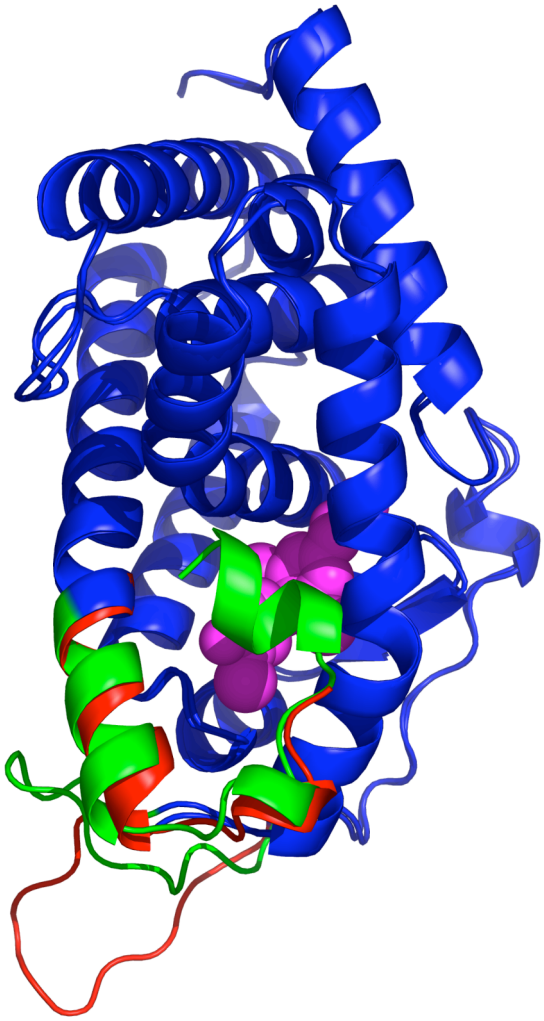

Figure 1-4: Structural alignment of v-erbA and rat thyroid hormone receptor α ligand binding domains.

A structural alignment of the rat thyroid hormone receptor a ligand binding domain (Wagner et al., 1995) and a homology model of the v-erbA ligand binding domain as determined via Modeller (Eswar et al., 2006). The C-terminal portion of TR is shown in green, while the C-terminal portion of v-erbA is shown in red.

Chapter

2

A Single Point Mutation in the Context of a Helix 12-Added v-erbA Restores Hormone Binding Ability

Introduction

An initial analysis of the nine point mutations in the v-erbA ligand binding domain was performed in order to determine which point mutations were most likely contributing to v-erbA's loss of ligand binding ability.

K186R and P191L: These mutations were not determined to be particularly interesting. Early work with v-erbA/TR chimeras suggested that these mutations do not contribute to the reduced ligand binding capacity of v-erbA (Steinmetz, Renaud, & Moras, 2001).

P203L: While located in the same loop as residues 186 and 191, was selected as having potential as an interesting mutant. The loop is located near the ligand binding pocket, and while residue 203 does not directly contact the hormone, it does appear to be located in an area which may have an effect on ligand binding capacity, with the P203L mutation possibly decreasing the LBD's affinity for T_3 by increasing the flexibility of this region of the loop.

K233N: The effect of this mutation on ligand binding was not immediately obvious, aside from the possibility of causing a shift in the position of helix 3, which forms a significant portion of the hormone binding pocket. This residue appears to be involved in capping helix 3, and thus a mutation at this position could have significant effects on the structure of this helix. This region of the LBD is also important for coactivator binding, and it is possible that this mutation might disrupt a salt bridge important in that interaction.

T342S: This mutation was not determined to be particularly interesting, as the position is highly variable among alpha and beta TR isoforms. Both human and rat TRβs have serines at this position, suggesting that this mutation would have little to no effect on v-ErbA ligand binding ability.

P363S, T370A and C378Y: These mutations appeared to be very interesting, as Helix 11 (the longest helix in TR) plays a crucial role in forming the hormone binding pocket. While these mutations are at the opposite end of Helix 11 from the ligand-contacting residues, they may cause a shift in the helix that

could have profound effects on ligand binding. In addition, Helix 11 forms the majority of the dimerization interface for the TR LBD, and the effects of these mutations may also manifest themselves in the alteration of these interactions.

F395S and Helix 12 Deletion (Residues 397-405): The importance of the F395S mutation is not immediately obvious and its effects may be related to those of the helix 12 deletion. The deletion of Helix 12 in the corresponding human TRβ F395X (notated as F451X in the human TRβ numbering scheme) mutant has been shown to have a significant effect on ligand binding, but v-erbA is much more severely compromised in ligand binding ability than F395X. It is interesting to note that the C-terminal tail of TRα, Q406-V408, which is deleted in F395X, is present in v-erbA. Given the large hydrophobic pocket in TRs, it seems quite possible that this tail may be interacting with the v-erbA binding pocket region, hindering ligand entry.

Ultimately, five point mutations were selected for study in the context of a Helix 12 addition to the v-erbA ligand binding domain: residues 203, 363, 370, 378, and 395 (Figure 2-1).

Materials and Methods

Vector constructs

The cDNAs encoding full-length chicken $TR\alpha_1$ (c-erbA) and gag-v-erbA in the eukaryotic expression vector pSG5 were obtained via generous gifts from

Martin L. Privalsky (University of California, Davis) (Yoh & Privalsky, 2001). The c-erbA and v-erbA ligand binding domain (LBD) cDNAs were created using polymerase chain reaction (PCR) and subcloned into the pET-15b bacterial expression vector (Novagen/EMD ?) using *Nde*I and *Bam*HI restriction sites. Helix 12 of c-erbA (residues 397-405) was inserted into the v-erbA LBD construct by BioMeans, Inc. (Sugar Land, TX) using *Sac*I and *Bam*HI restriction sites. Amino acid substitutions were created using the QuikChange Site-Directed Mutagenesis Kit (Strategene/Agilent Technologies, La Jolla, CA).

Protein expression

All constructs were expressed in BL21(DE3) cells grown at 37°C to OD_{600}=0.8, followed by induction for approximately 18 hours at 16°C using 500 μM isopropylthiogalactoside (IPTG). The cultures were subsequently centrifuged at 10,000×*g* for 15 minutes, and cell pellets were either used fresh for purification or flash frozen in liquid nitrogen and stored at -80°C.

Purification

For all constructs, cell pellets were resuspended in lysis buffer consisting of 20 mM Hepes pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 0.2 mM Tris(2-Carboxyethyl) phosphine (TCEP), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 25 mM imidazole and one Complete, EDTA-free protease inhibitor tablet (Roche Diagnostics GMBH, Mannheim, Germany) per 50 ml of lysis buffer. Resuspension was carried out 15 ml of lysis buffer per liter of cell culture, with 10 mg egg white lysozyme (Thermo Fisher Scientific, Pittsburgh, PA) and **DNase**

added to the cell pellets just prior to buffer addition. Resuspended cells were disrupted and homogenized using a Sonifer S-250 (Branson Ultrasonics Corporation, Danbury, CT).

Constructs were initially purified via 6xHis affinity with Ni-NTA beads (QIAGEN N.V., Venlo, The Netherlands) using 3 ml of slurry per liter of cell culture. After washing beads with 10 ml of lysis buffer, lysed cells and beads were mixed for one hour at 4°C. The beads were subsequently washed twice in 10 ml of lysis buffer each and then transferred to a Poly-Prep chromatography column (Bio-Rad Laboratories, Inc., Hercules, CA) and washed three additional times with 10 ml of lysis buffer each. Elution was performed using an elution buffer identical to the lysis buffer except for an increased imidazole concentration of 350 mM. Six 1-ml elution fractions were obtained with fractions 2-6 utilized for further purification.

Fractions 2-6 were combined and dialyzed overnight using a 3-12 ml capacity Slide-A-Lyzer 10K molecular weight cutoff dialysis cassette into a gel filtration buffer consisting of 20 mM Hepes pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 0.2 mM TCEP, 3 mM dithiothreitol (DTT), and 1 mM ethylenediaminetetraacetic acid (EDTA). Following dialysis, samples were concentrated using an Amicon Ultra-15 centrifugal filter unit (Millipore Corporation, Bellerica, MA) to approximately 500 µl and injected onto a HiLoad Superdex 75 16/60 column connected to a Pharmacia FPLC system. Separation was performed at a flow rate of 0.5 ml per minute, and 2-ml fractions were collected. The desired peak was collected and concentrated to approximately 15

mg/ml. Protein samples were either used fresh for subsequent experiments or flash frozen in liquid nitrogen and stored at -80°C for later use.

T_3-binding assays

Thyroid hormone (T_3) binding assays were performed as described by Apriletti (Apriletti, Baxter, Lau, & West, 1995) and the equilibrium dissociation constant (K_d) values were calculated from the competition data using the Prism computer program (GraphPad Software, Inc., La Jolla, CA).

Assay for partial elastase proteolysis

Proteolytic digestions were performed in a final volume of 100 μl containing 20 mM Hepes (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.2 mM Tris(2-Carboxyethyl) phosphine (TCEP), 10% (v/v) glycerol, 50 μg of purified protein LBD construct, and 100 ng of elastase (Boehringer Mannheim, Indianapolis, IN). Four LBD constructs were subjected to digestion: c-erbA, v-erbA, v-erbA + Helix 12, and v-erbA + Helix 12 (S395F). Samples were incubated at 25°C, and aliquots (10 μl each) were taken at 0, 5, 15, 30, 60, and 120 min. Proteolysis was quenched by the addition of 4X loading buffer and incubation at 95°C for 5 min. Samples were analyzed by SDS-PAGE using 10% Bis-Tris gels.

Unfolding temperature determination using differential scanning fluorimetry

Unfolding temperatures for the c-erbA, v-erbA, v-erbA + H12, and v-erbA + H12 (S395F) were obtained in the presence and absence of Triac using differential scanning fluorimetry (Pantoliano et al., 2001) (Lo et al., 2004). A 96-

well deep-well plate was used with a final volume of 50 μl per well containing 10 μg of protein. For assays conducted in the presence of hormone, Triac was included at a 3X molar excess. All samples utilized SYPRO Orange (Molecular Probes/Invitrogen, Eugene OR) as the environmentally-sensitive fluorescent dye. Samples were exposed to an increase of 2°C/min from an initial temperature of 25°C to a final temperature of 95°C. The increase in fluorescence as the dye bound to hydrophobic regions of unfolding proteins allowed for calculation of the melting temperatures based on the inflection point of the fluorescence curve.

Transcriptional assays

HeLa cells were stably transfected with the desired full-length construct (10 μg per assay well) and a luciferase reporter gene (200 μg per assay well). This reporter gene contained a thyroid hormone response element (TRE) utilizing a direct repeat of the consensus TR DNA-binding site (DR4 element). Assays were performed as described by Webb (Webb et al., 2000) and Velasco (Velasco et al., 2007).

Experimental Results

Binding of T3 to c-erbA, v-erbA, and v-erbA mutants

The abilities of each of eight LBD constructs to bind T_3 in competition assays with [^{125}I]T_3 are shown in Figure 2-2 and Table 2-1 (also see *Materials and Methods*). Using non-linear regression analysis, the K_d for T_3 binding to the c-erbA LBD was calculated to be 3.2 nM. The K_ds for the v-erbA LBD, v-erbA LBD + Helix 12, and four of the five additional point mutants were approximately

1000-fold higher. The fifth point mutant, S395F, demonstrated a K_d of 33 nM, only 10-fold higher than for the c-erbA LBD.

Partial proteolysis

The resistance of several LBD constructs to elastase degradation in the absence and presence of T_3 was determined using SDS-PAGE and Coomassie staining as shown in Figure 2-3. The c-erbA LBD was remarkably stable over the 120-minute incubation, exhibiting essentially no observable degradation in the presence of T_3 and only minor degradation in the absence of T_3. The v-erbA LBD and v-erbA LBD + Helix 12 constructs demonstrated greatly reduced resistance to degradation, as significant quantities of degradation product were visible on the gel after only five minutes of incubation with elastase. The v-erbA + Helix 12 S395F point mutant demonstrated increased resistance compared to the other v-erbA-based constructs, with significant degradation no appearing until 15 minutes into the incubation, but was less resistant than the c-erbA LBD.

Differential scanning fluorimetry

The stability of several LBD constructs was assayed using differential scanning fluorimetry (see *Materials and Methods*). All constructs demonstrated higher T_ms in the presence of T_3 than in the absence of hormone. The c-erbA LBD construct yielded the highest T_ms at 61.3°C in the presence of hormone and 58.5°C in the absence of hormone. The S395F point mutant exhibited intermediate T_m values of 57.3°C in the presence of hormone and 55.0°C in the absence of hormone. Both the v-erbA LBD and v-erbA LBD + Helix 12 constructs

exhibited lower T_m values, with v-erbA having the lowest T_ms at 54.0°C and 52.5°C and v-erbA + Helix 12 having T_ms of 55.0°C and 54.0°C.

The results of these experiments are shown in Figures 2-4, 25-, 2-6 and 2-7 and Table 2-2.

Transcriptional modulation by c-erbA, v-erbA, and v-erbA mutants

The ability of each of the four primary constructs to mediate a thyroid hormone transcriptional response was tested in cell culture. In these experiments, HeLa cells were stably transfected with the desired full-length construct (10 μg per assay well) and a luciferase reporter gene (200 μg per assay well). This reporter gene contained a thyroid hormone response element (TRE) utilizing a direct repeat of the consensus TR DNA-binding site (DR4 element).

The results demonstrated repression over basal transcriptional levels for all constructs in the absence of hormone, and varying degrees of activation in the presence of hormone. While human and chicken TR constructs demonstrated significant activation above basal levels, v-erbA and v-erbA + Helix 12 constructs showed no activation, and in fact maintained repression of transcription even in the presence of hormone. The v-erbA + Helix 12 S395F mutant, however, did exhibit transcriptional activation at levels 2-3 times that of the wild-type TR constructs.

Results of a single-concentration activation and repression expression at a hormone concentration of 1 μM are shown in Figure 2-8. Activation curves for concentration series ranging logarithmically from 100 pM to 1 μM are shown in Figure 2-9.

Discussion

These studies demonstrate the profound effect of an S395F v-erbA mutant in the presence of a Helix 12 addition. While previous work has shown that the addition of Helix 12 alone to v-erbA was not sufficient to restore ligand binding, protein stability, and transcriptional activity to near wild-type c-erbA levels, the effects of an additional reversion mutation at position 395 had not been explored prior to this work.

In this work, it was determined that the binding constants measured using [^{125}I]T_3 competition assays on LBD constructs reveal that the addition of Helix 12 does not appreciably increase the v-erbA LBD's hormone-binding ability, an expected result. While the wild-type c-erbA LBD construct in the presence of T_3 exhibits a K_d of 3.2 nM, both the v-erbA LBD and the v-erbA LBD plus Helix 12 both exhibit K_ds in the 3-4 μM range, for a 1000-fold decrease in hormone binding ability. The results for four of the v-erbA LBD point mutations (L203P, S363P, A370T, Y378C) tested individually within the context of the Helix 12 addition demonstrated no gain in hormone binding ability.

The fifth v-erbA LBD point mutation, a serine-to-phenylalanine mutation at position 395 slightly N-terminal to Helix 12 yielded a K_d of 33 nM, demonstrating a 100-fold increase in binding affinity over other v-erbA LBD constructs, and thus only 10-fold compromised hormone binding ability when compared to the wild-type thyroid hormone receptor c-erbA LBD.

It was also determined that the S395F mutation in the context of a Helix 12 addition also increases protein stability relative to wild-type v-erbA. Both

qualitative partial proteolysis and quantitative thermal stability assays suggest stability for the H12 (S395F) construct midway between that of v-erbA/v-erbA+H12 and c-erbA. Furthermore, both assays reveal that the addition of T_3 hormone increases protein stability for all constructs. Interestingly, the differential scanning fluorimetry results suggest that the v-erbA and v-erbA+H12 constructs are particularly unstable at low temperatures, with increasing stability possibly occurring with an increase in temperature. Alternatively, decreasing signal in response to increasing temperature could signify aggregation of unstable proteins. Regardless, a sufficient amount of protein remains properly folded at low temperatures that a melting transition can be observed.

Finally, transcriptional experiments extending the results to full-length constructs in cell culture are consistent with the behavior observed for LBD constructs *in vitro*. Control experiments containing only an empty pCMV-Tag2B vector revealed similar basal levels of luciferase activity in both the presence and absence of hormone. Addition of various full-length v-erbA and c-erbA constructs to the pCMV-Tag2B vector, however, revealed significant differences in activity.

Both human and chicken TRα demonstrated prototypical TR behavior in these assays as expected. In the absence of hormone, each receptor was able to repress transcriptional activity, as evidenced by significantly decreased luciferase activity when compared to the control experiments using the empty vector. In the presence of hormone, however, each receptor activated transcription, leading to increased luciferase activity.

In the cases of v-erbA and v-erba+H12, a different pattern of activity was observed. In the absence of hormone, transcriptional activity was reduced when compared to empty vector controls. The amount of reduction in activity was similar to that seen in the wild-type TR constructs. However, addition of hormone to these constructs not only failed to activate transcription, but also failed to lessen the repression observed in the absence of hormone. Both the v-erbA and v-erbA+H12 constructs appear to be completely insensitive to hormone when assayed for transcriptional ability.

The final construct, v-erbA+H12 (S395F) yielded surprising results, considering that it possesses only a single amino acid residue difference when compared to the v-erbA+H12 construct. In the absence of hormone, v-erbA+H12 (S395F) was able to repress transcriptional activity on par with both c-erbA and other v-erbA constructs. However, addition of hormone resulted in a significant increase in transcriptional activity, demonstrating approximately as much increase in luciferase activity above basal control levels as chicken TRα.

In response to the results of the single-concentration experiments, additional experiments were undertaken to assess the behavior of a hormone concentration curve on transcriptional activity. The experiments were performed using the exact same constructs as in the single-concentration assay, although human TRα was not included in the study. All four constructs again demonstrated the ability to repress transcription when compared to an empty pCMV-Tag2B vector. As expected, neither the v-erbA construct nor the v-erbA+H12 construct was able to activate transcription even at hormone levels up to 1 μM.

The c-erbA construct, on the other hand, began activating transcription at a hormone concentration of approximately 1 nM, showing modest increases in transcriptional activity with hormone concentration topping out at approximately twice the basal levels at a hormone concentration of 1μM. The v-erbA+H12 (S395F) construct required a higher hormone concentration in excess of 10 nM before reporter activity was increased above basal control levels, but activation increased much more sharply than with c-erbA, reaching three-fold increase over basal activity at 1μM. Both of these results are consistent with other data showing both a 10-fold lower hormone binding affinity for the v-erbA+H12 (S395F) LBD when compared to c-erbA LBD and a significantly greater transcriptional activation of the luciferase gene at a hormone concentration of 1 μM for the full-length v-erbA+H12 (S395F) construct over the corresponding full-length c-erbA construct.

Summary

In summary, this work demonstrated several pieces of evidence that residue 395 of the thyroid hormone receptor plays an important role in the receptors ability bind hormone and regulate transcription.

First, work with constructs containing only the ligand binding domains of c-erbA and various v-erbA mutations demonstrated that while addition of Helix 12 to v-erbA did not significantly restore ligand binding ability to v-erbA, an additional single point mutation at residue 395 (S395F) increased the binding affinity within ten-fold of wild-type c-erbA.

Second, both differential scanning fluorimetry and protease stability assays demonstrated that the S395F mutation significantly enhances protein stability when compared to wild-type v-erbA or v-erbA with Helix 12 added, although the protein did not match the stability observed with wild-type c-erbA.

Third, transcriptional data obtained using full-length constructs in transient transfection assays demonstrated that the S395F mutation significantly enhance the ability of v-erbA to activate transcription in the presence of hormone. While both v-erbA and v-erbA + Helix 12 constructs not only failed to activate transcription in the presence of hormone but maintain repression seen in the absence of hormone, the v-erbA + Helix 12 S395F mutant activated transcription even beyond the level seen with wild-type c-erbA.

Future Work

The results of these experiments highlighting the importance of residue 395 in hormone binding, protein stability, and transcriptional control suggest a number of future experiments. Of significant interest to a structural biology laboratory would be a three-dimensional structure in atomic detail of several of these constructs comparing the effects of the point mutation at residue 395 on the positioning of Helix 12 with respect to formation of both the hydrophobic hormone binding pocket in the interior of the receptor as well as the exterior binding surface for coactivator proteins involved in transcription. Early efforts toward this goal are described in Chapter 3.

A second target of follow-up research includes approaching this same question from the opposite perspective, that of c-erbA. Conducting many of these

same experiments with full-length and LBD c-erbA constructs containing an F395S point mutation should yield corresponding results in which the hormone binding ability, protein stability, and transcriptional control of c-erbA would be negatively affected. This approach in the absence of other v-erbA mutations would confirm the importance of residue 395 to the overall function of the thyroid hormone receptor. Initial steps toward this goal were taken, but cloning difficulties related to a poly-G region of the DNA at the location of this residue, as well as time constraints, prevented its inclusion in this work.

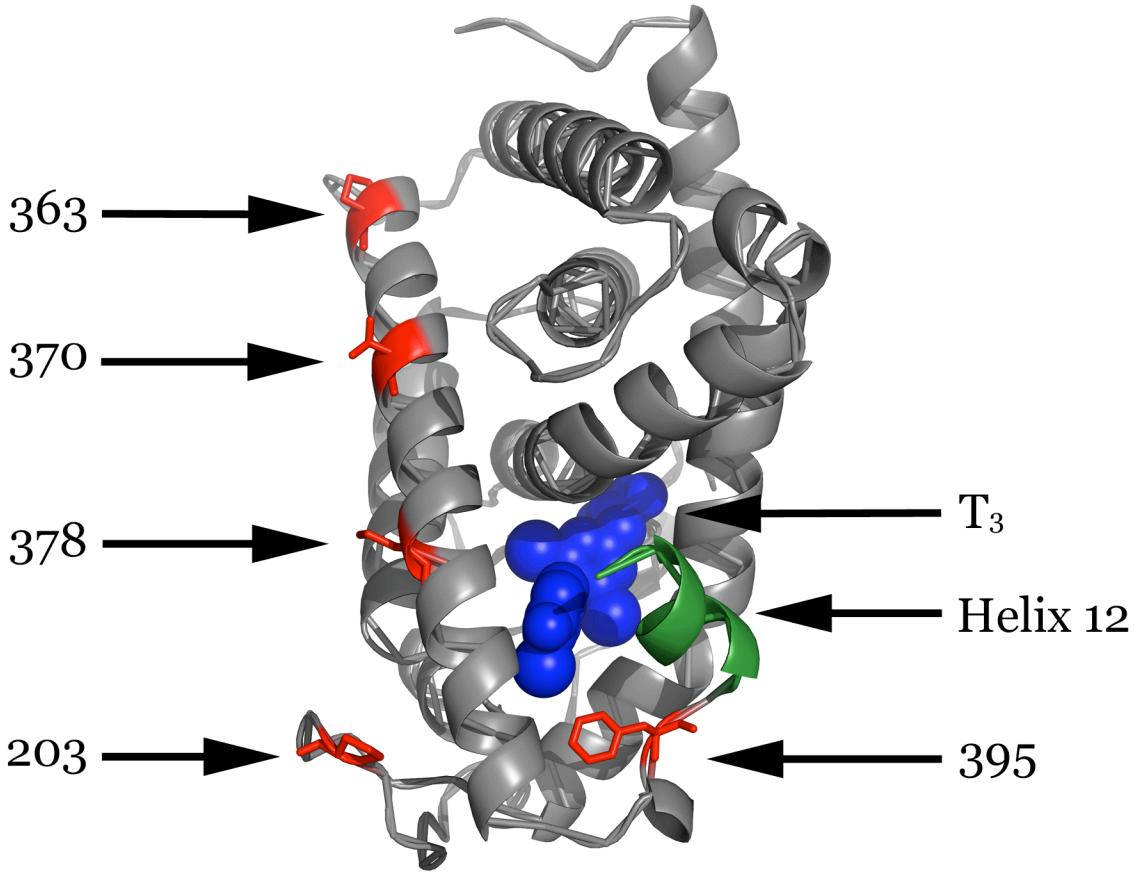

Figure 2-1: Crystal structure overview of point mutations

Crystal structure of rat thyroid hormone receptor α ligand binding showing the locations of hormone, Helix 12, and the five point mutations addressed in this work.

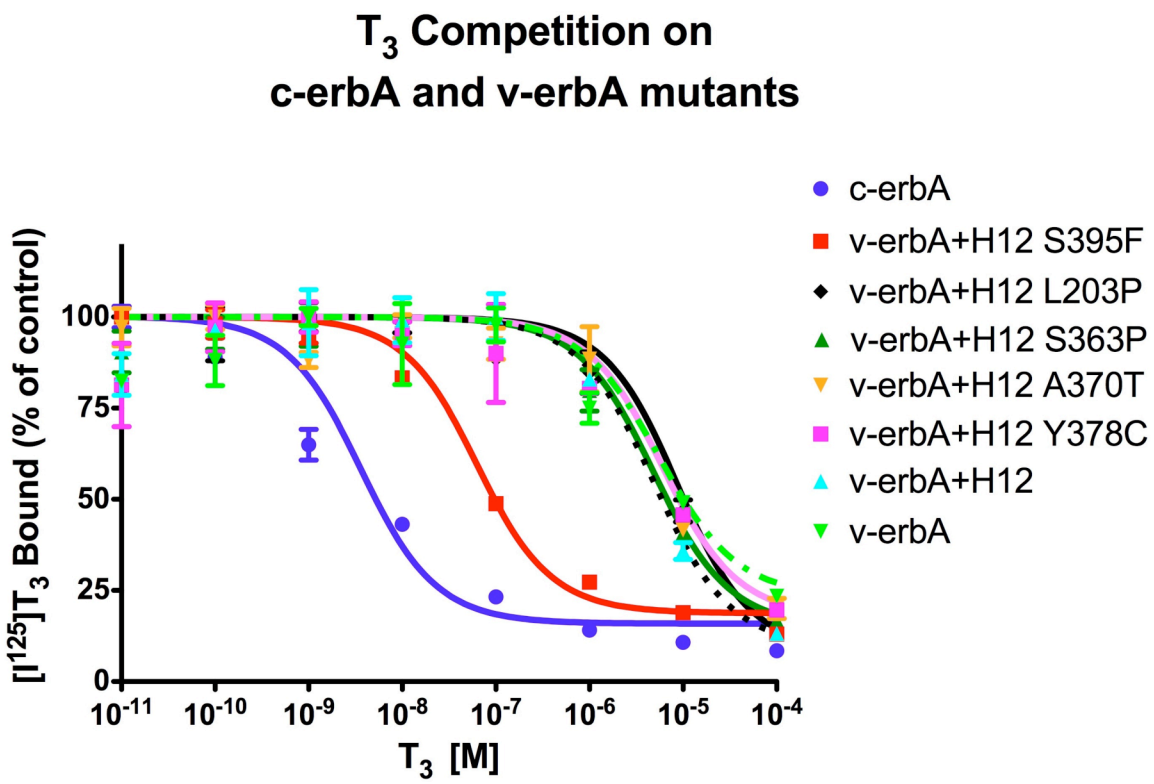

Figure 2-2: T_3 hormone binding curves for c-erbA, v-erbA, and mutants

Binding curves for the $[I^{125}]T_3$ hormone binding assays demonstrating tight binding by c-erbA, ten-fold compromised binding by v-erbA+H12 S395F, and poor binding by the remaining constructs.

Construct	K_d
c-erba	**3.1 nM**
S395F	**33 nM**
v-erbA	4.2 μM
v-erbA+H12	3.2 μM
L203P	4.7 μM
S363P	3.1 μM
A370T	4.0 μM
Y378C	3.5 μM

Table 2-1: Dissociation constants for T_3 hormone binding by c-erbA, v-erbA, and mutants

The wild-type chicken thyroid hormone receptor a, c-erbA, bound hormone with a K_d of 3.2 nM. While v-erbA, v-erbA + Helix 12, and four of the five point mutations in the context of the Helix 12 addition all demonstrated approximately 1000-fold compromised ligand binding ability, the v-erbA + Helix 12 S395F construct demonstrated only ten-fold compromised binding with a K_d of 33 nM.

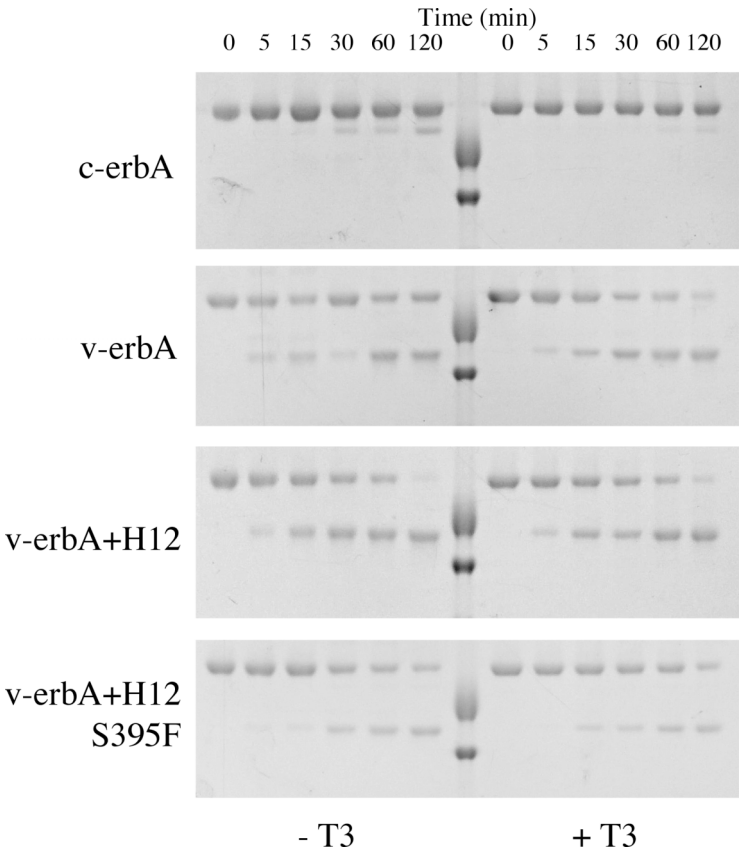

Figure 2-3: Partial elastase proteolysis of c-erbA and v-erbA ligand binding domain constructs.

SDS-PAGE results illustrate protein degradation over increasing time of exposure to elastase.. All four constructs demonstrate increased stability in the presence of hormone. The c-erbA LBD is the most stable of the four constructs, with the v-erbA +H12 S395F mutant exhibiting somewhat less stability, as evidence by the degradation product beginning to appear after approximately 15 minutes. The v-erbA and v-erbA+H12 constructs are the least stable, showing significant degradation after only 5 minutes.

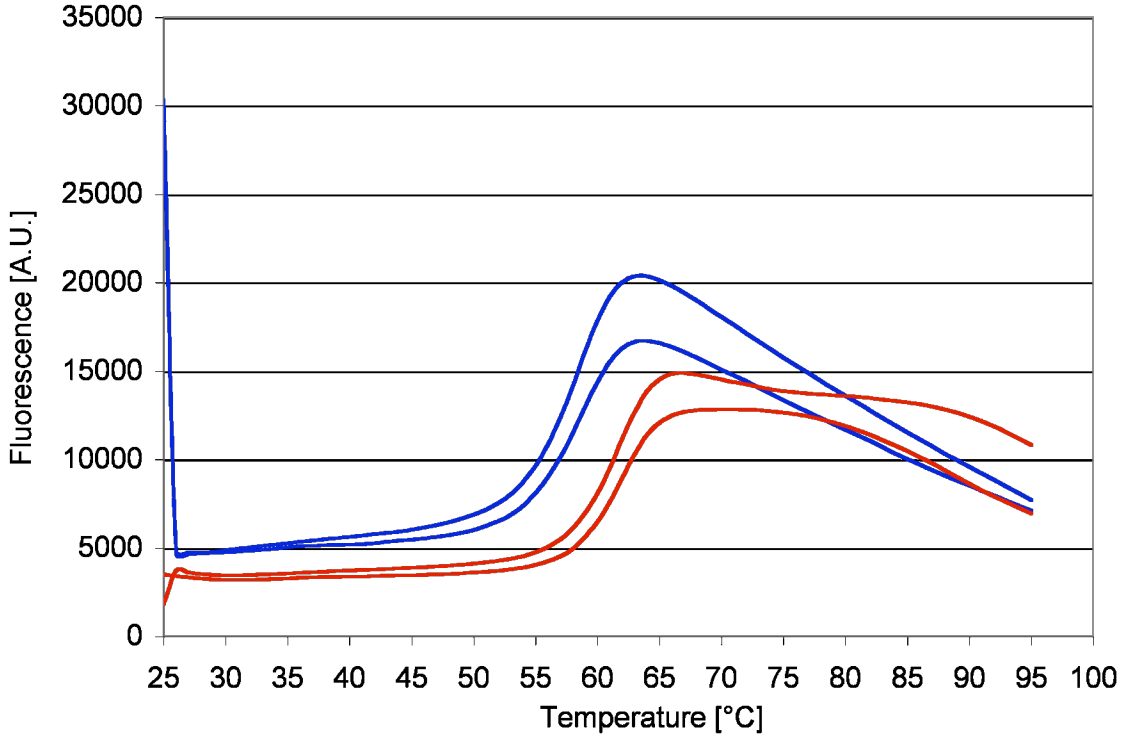

Figure 2-4: Melting temperature data for c-erbA LBD

Melting temperature curves for c-erbA LBD reveal an increase in T_m for c-erbA in the presence of hormone (red lines) when compared to the absence of hormone (blue lines). c-erbA behavior in the assays is typical of well-folded proteins.

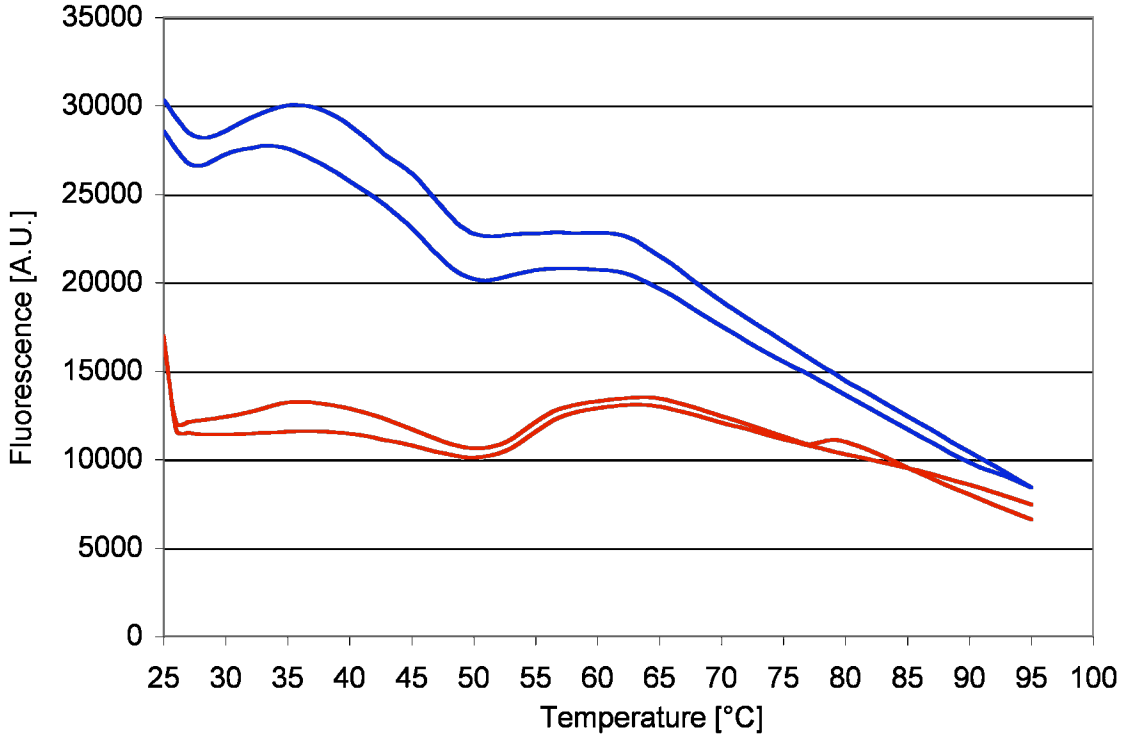

Figure 2-5: Melting temperature data for v-erbA LBD

Melting temperature curves for v-erbA LBD reveal an increase in T_m for v-erbA in the presence of hormone (red lines) when compared to the absence of hormone (blue lines). High signal at low temperatures is indicative of an unstable protein with a strong tendency to aggregate.

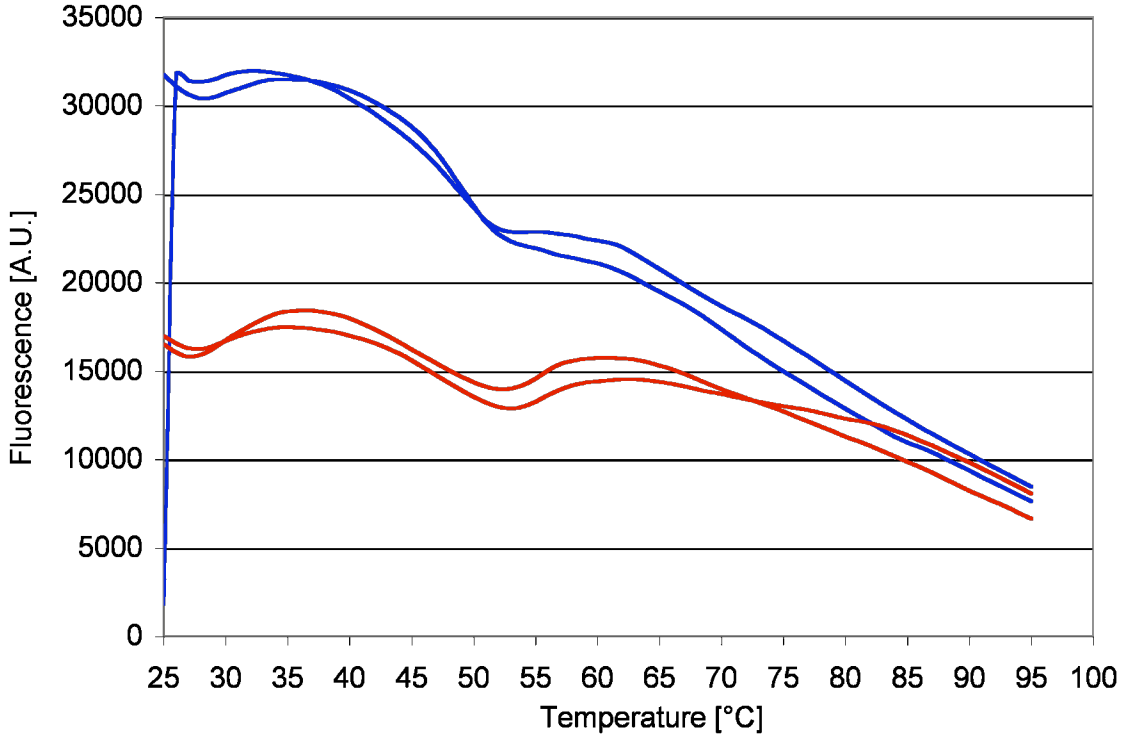

Figure 2-6: Melting temperature data for v-erbA + Helix 12 LBD

Melting temperature curves for v-erbA + Helix 12 LBD reveal an increase in T_m for v-erbA + Helix 12 in the presence of hormone (red lines) when compared to the absence of hormone (blue lines). High signal at low temperatures is indicative of an unstable protein with a strong tendency to aggregate.

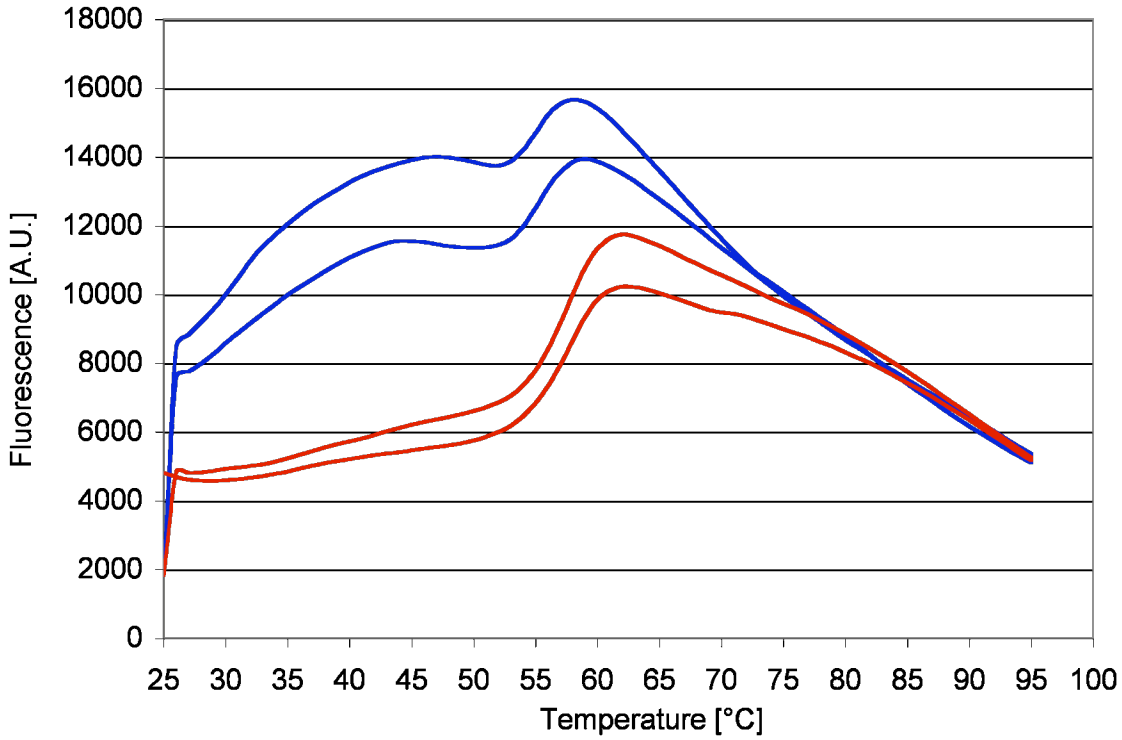

Figure 2-7: Melting temperature data for v-erbA + Helix 12 S395F LBD

Melting temperature curves for v-erbA + Helix 12 S395F LBD reveal an increase in T_m for v-erbA + Helix 12 S395Fin the presence of hormone (red lines) when compared to the absence of hormone (blue lines). The curves demonstrate a composite of stability behavior observed in the other constructs. High signal at low temperatures in the absence of hormone is indicative of an unstable protein with a strong tendency to aggregate. In the presence of hormone, however, the construct is considerably more well-folded and well-behaved.

Construct	No T_3	T_3
c-erba	**58.5°**	**61.3°**
v-erbA	52.5°	54.0°
v-erbA+H12	54.0°	55.0°
S395F	**55.0°**	**57.3°**

Table 2-2: Melting temperatures for LBD constructs in presence and absence of T_3

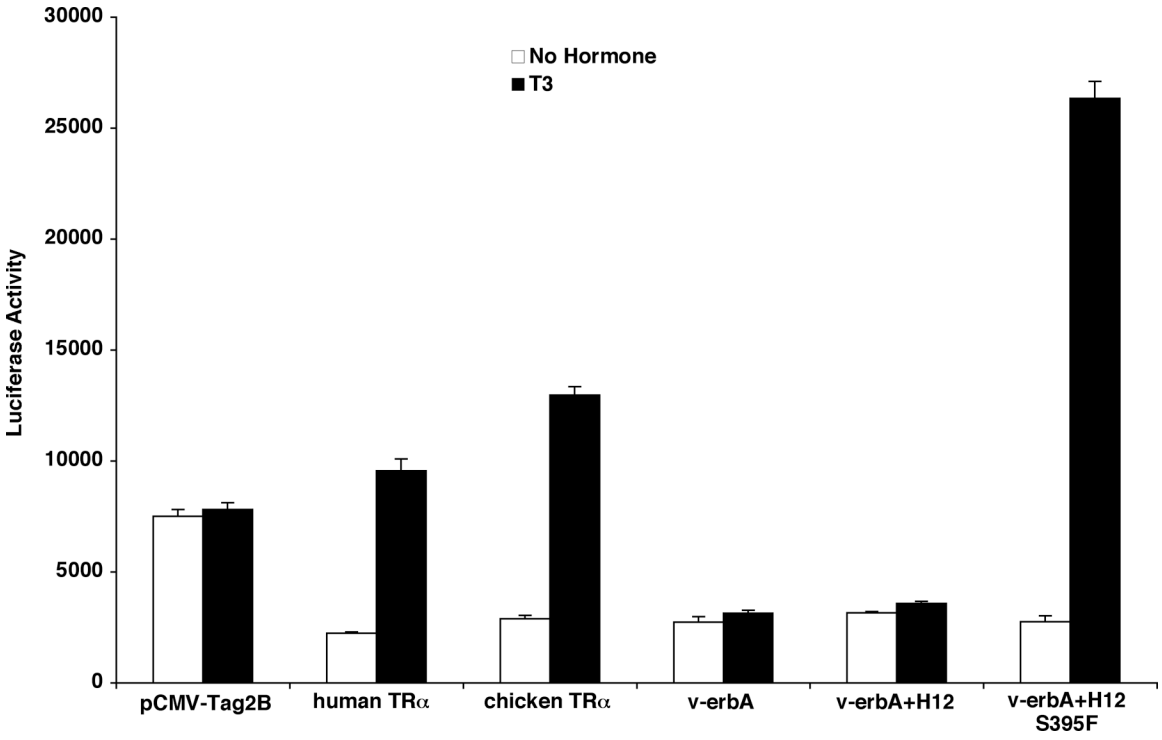

Figure 2-8: Single-concentration transient transfection luciferase assay for transcriptional control

In the absence of hormone, all assayed receptors effectively repress basal levels of luciferase transcription. At a concentration of 1 μM T_3, wild-type human and chicken TRα activates transcription, while v-erbA and v-erbA + Helix 12 are unresponsive and continue to repress transcription. The addition of an S395F mutation to the v-erbA + Helix 12 construct reverses this behavior resulting in effective activation of transcription even greater than that of wild-type TRα constructs.

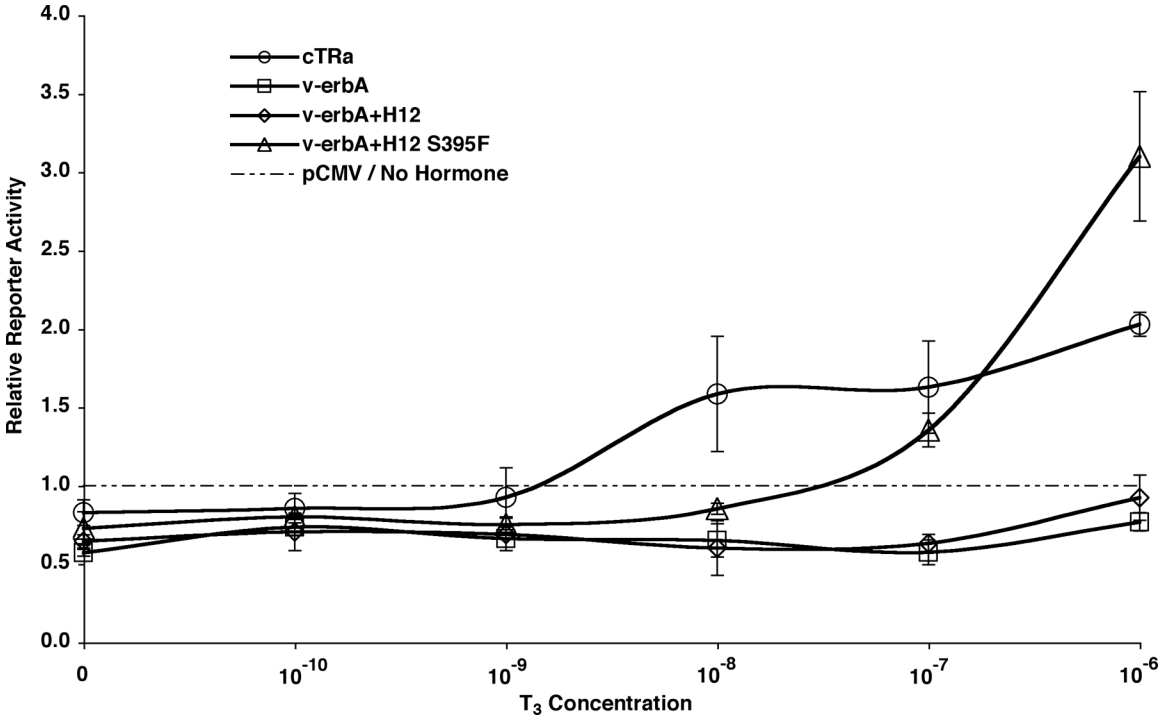

Figure 2-9: Concentration series for transient transfection luciferase assay for transcriptional control

At low concentrations of hormone, all assayed receptors effectively repress basal levels of luciferase transcription. As T_3 concentration reaches 1 nM, wild-type chicken TRα begins to activate transcription, increasing twice basal levels by 1 μM. Conversely, v-erbA and v-erbA + Helix 12 are unresponsive and continue to repress transcription, showing only the slightest easing of repression as T_3 concentration reaches 1 μM. The addition of an S395F mutation to the v-erbA + Helix 12 construct resulting in effective activation of transcription even greater than that of wild-type TRα constructs, although a higher concentration of T_3 on the order of 10 μM is required in order to begin activation of transcription.

CHAPTER

3

Preliminary Crystallographic Studies of Thyroid Hormone Receptor and v-erbA

Introduction

High-resolution crystal structures of a number of nuclear receptor ligand-binding domains (Bourguet, Ruff, Chambon, Gronemeyer, & Moras, 1995; Brzozowski et al., 1997; Nolte et al., 1998; Renaud et al., 1995; Wagner et al., 1995; Williams & Sigler, 1998) and DNA-binding domains have been determined. However, at the time of this project, no multi-domain crystal structure had yet been solved. Consequently, a project was undertaken to solve the crystal structure.

Additional crystallography projects were undertaken in order attempt to understand the dimerization interface of the thyroid hormone receptor and the retinoid x receptor and to use v-erbA as a structural model for unliganded thyroid hormone receptor.

Experimental Results and Discussion

Unliganded TRβ DBD-LBD homodimer in complex with DNA and corepressor peptide

Crystal trials of a large complex containing a homodimer of a human TRβ DBD-LBD construct bound to a 26-residue peptide from the corepressor NCoR and a 21-base pair double-stranded oligonucleotide containing two thyroid hormone receptor response elements arranged in an F2 everted repeat configuration yielded a single preliminary hit. The hit was discovered in Nextal Classics Lite condition #69, consisting of 0.05M potassium phosphate monobasic and 10% w/v PEG 8000 (Figure 3-1).

Assessment of the crystals found in the condition using a Korima fluorescence microscope suggested that the crystals were indeed protein and not salt. However, screening of the crystals at Beamline 8.3.1 at the Advanced Light Source in Berkeley, California did not yield positive results. One crystal demonstrated no diffraction, while the second yielded a few extremely low-resolution diffraction spots.

Extensive attempts to reproduce crystals in the initial condition, as well as screens around the initial condition, proved unsuccessful, and the project was ultimately placed on indefinite hold. Several years after the project was

abandoned, the first multi-domain structure of a nuclear receptor was determined with PPARγ and RXRα bound to DNA (Figure 3-2) (Chandra et al., 2008).

Liganded TRβ -RXRα LBD heterodimer bound to ligands and coactivator peptide

Previous crystal trials had generated hits on a complex of TRβ LBD bound to Triac, RXRα bound to several different ligands, and a 13-residue peptide from the coactivator GRIP-1 with sequence KHKILHRLLQDSS. These hits were discovered in Hampton PEG/Ion condition #25, consisting of 0.2M magnesium acetate and 20% w/v PEG 3350, and had demonstrated x-ray diffraction to 9Å.

Further screening studies were undertaken utilizing Hampton detergent and additive screens, which yielded several different crystal forms (Figure 3-3) in several different conditions, with 1-s-nonyl-β-d-thioglucoside appearing to best promote crystal growth. Additional screening using Nextal OptiSalts in conjunction with 1-s-nonyl-β-d-thioglucoside yielded further crystal growth. Preliminary x-ray studies on these crystals yielded diffraction to approximately 7Å.

Optimization of crystal growth continued, finally seeing diffraction to approximately 2.7Å, and a dataset was collected at the Advanced Light Source Beamline 8.3.1. (Table 3-1) Unfortunately, solving the structure via molecular replacement revealed that only the TR LBD and peptide were present in the crystal. (Figure 3-4) The same structure had been determined to a resolution of

3.6Å several years earlier (Dairmont et al., 1998), so this project was placed on hold in favor of other, more promising projects.

v-erbA LBD bound to corepressor peptide

Numerous crystal trays were set up using both v-erbA LBD and v-erbA LBD + Helix 12 constructs. Hanging drops were set with the two constructs both in the presence and absence of a corepressor peptide. No crystal hits were discovered in any of the conditions tested, and the project was eventually abandoned in order to focus on biochemical experiments.

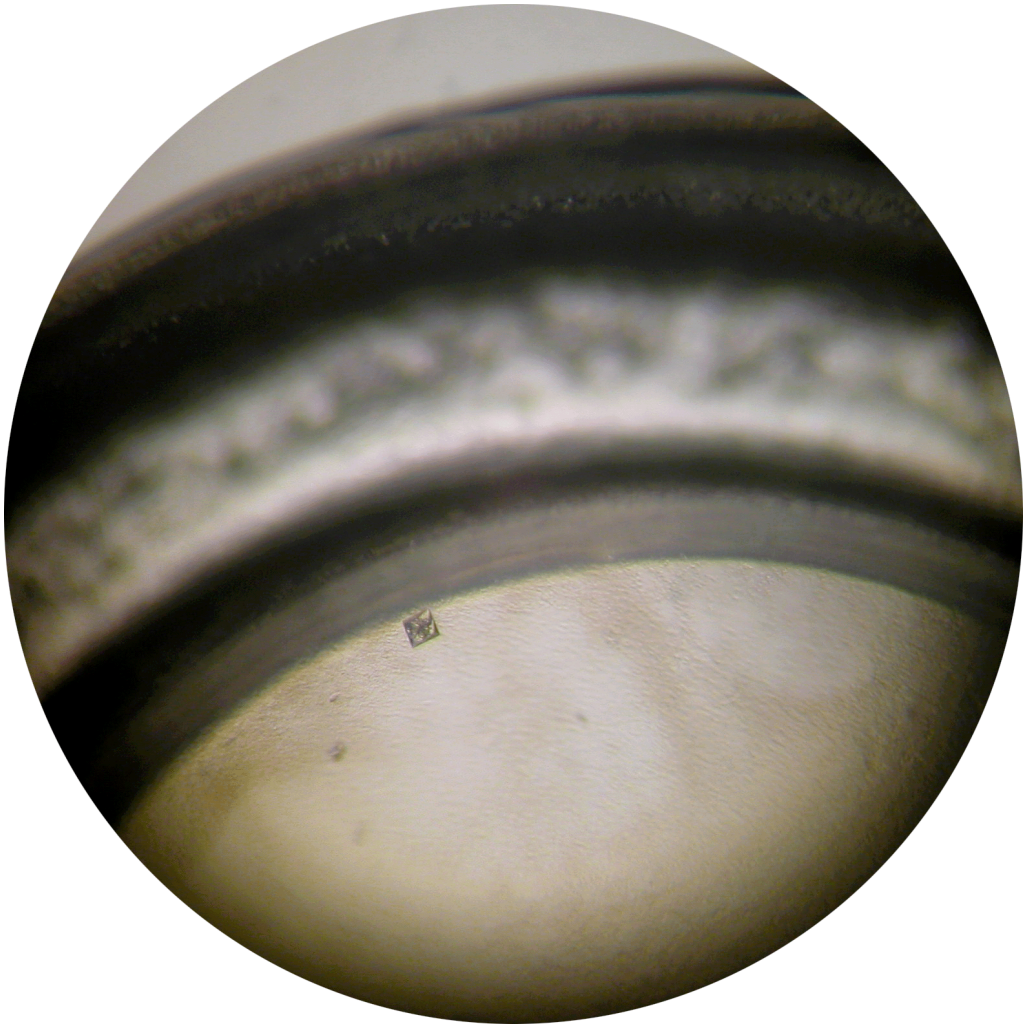

Figure 3-1: Microscope image of crystal obtained from unliganded TRβ DBD-LBD homodimer in complex with DNA and corepressor peptide

Crystal hit obtained from a drop of TRβ DBD-LBD homodimer in complex with DNA and corepressor peptide in Nextal Classics Lite condition #69, consisting of 0.05M potassium phosphate monobasic and 10% w/v PEG 8000.

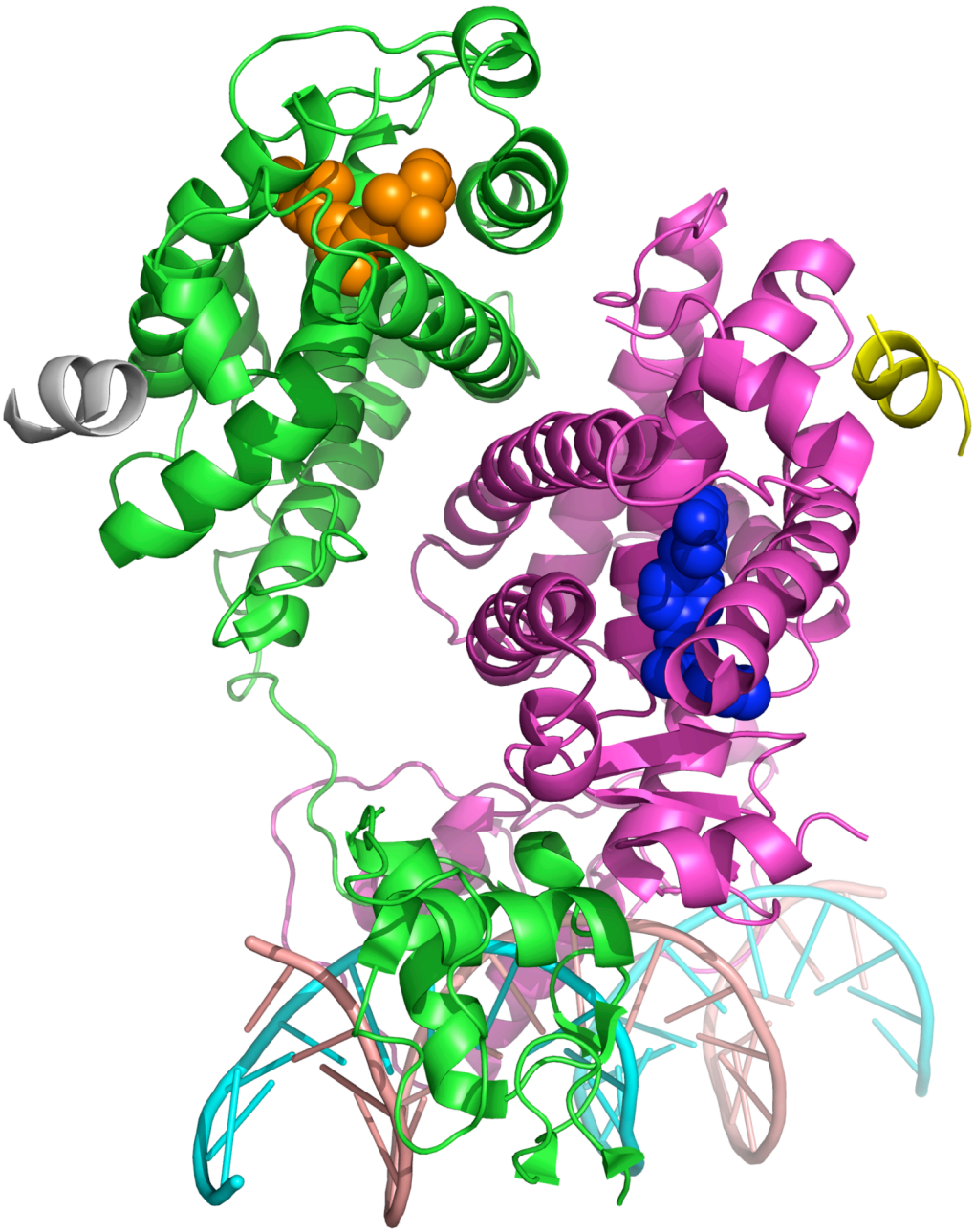

Figure 3-2: Crystal structure of PPARγ - RXRα nuclear receptor complex on DNA

First crystal multi-domain structure of a nuclear receptor, PPARγ and RXRα bound to DNA (Chandra et al., 2008).

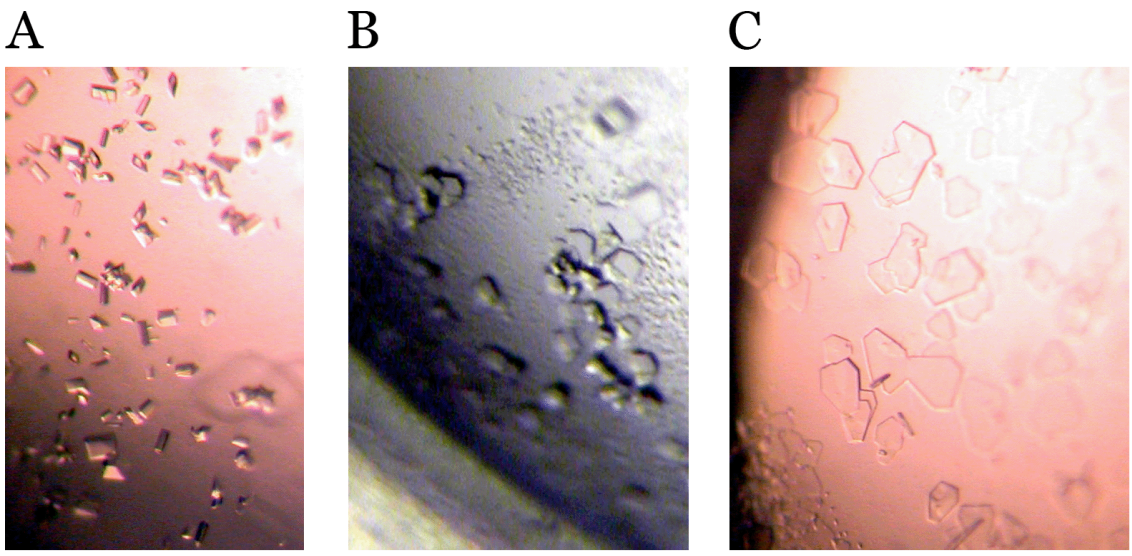

Figure 3-3: Microscope images of crystals obtained liganded TRβ - RXRα LBD heterodimer bound to ligands and coactivator peptide

Multiple crystals obtained from liganded TRβ -RXRα LBD heterodimer bound to ligands and coactivator peptide diffracting to 7Å at best. Crystals shown in Panel A utilized 9-*cis*-retinonic acid as the RXR ligand, while Panel B and Panel C show two different crystal forms obtained using methoprene acid as the RXR ligand.

space group	$P3_121$
unit cell constants	
a	87.131 Å
b	87.131 Å
c	65.678 Å
α	90.0°
β	90.0°
γ	120.0°
highest resolution (Å)	2.7
unique reflections	7965
completeness	97.4%
solvent content	45.8%
refinement resolution range (Å)	25.0 - 2.7
R	33.7%
free R	41.4%
water molecules	5
rmsd in bond lengths (Å)	0.011
rmsd in bond angles (deg)	1.4

Table 3-1: Crystallographic statistics of TRβ LBD with GRIP-1 peptide

Data set obtained at Advanced Light Source Beamline 8.3.1 initially thought to be liganded TRβ -RXRα LBD heterodimer with GRIP-1 peptide, but upon solution was determined to be only TRβ LBD with GRIP-1 peptide, a structure determined previously.

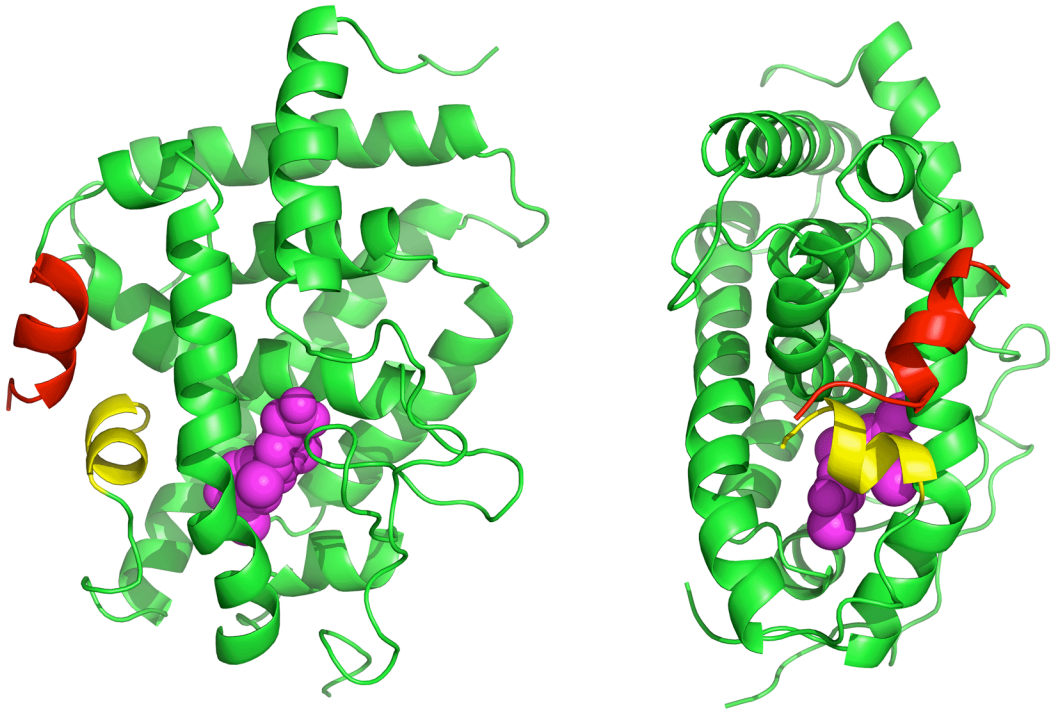

Figure 3-4: Crystal structure of TRβ LBD with GRIP-1 peptide

Crystal structure determined from data obtained at Advanced Light Source Beamline 8.3.1 initially thought to be liganded TRβ -RXRα LBD heterodimer with GRIP-1 peptide, but upon solution was determined to be only TRβ LBD with GRIP-1 peptide, a structure determined previously. The thyroid hormone Triac is shown in purple, with Helix 12 shown in yellow and the GRIP-1 peptide shown in red.

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