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Characterization of the Molecular Mechanisms Regulating the Transcriptional Activity of the RORα Orphan Nuclear Receptor

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Abstract

Nuclear receptors are transcription factors that regulate gene expression in response to small lipophilic molecules, including steroid hormones, thyroid hormone, and vitamin A and D metabolites. RORα is an orphan nuclear receptor that was initially cloned based on its similarities with the retinoic acid receptor. The term orphan was coined in reference to a nuclear receptor whose discovery has preceded that of its ligand. Genetic ablation of the *Rora* gene in mice leads to severe cerebellar ataxia, known as the *staggerer* phenotype. RORα regulates a myriad of genes involved in cellular differentiation, including myogenesis and adipogenesis, as well as various metabolic pathways. Nuclear receptors control the expression of their target genes by binding to short DNA sequences, referred to as hormone response elements, as monomers, homodimers, or heterodimers with the common partner RXR. RORα is strictly a monomeric DNA binding protein, lacking key molecular determinants in its DNA binding domain essential for cooperative homodimer formation. This orphan receptor is a potent transcriptional activator, whose activity is dependent on an endogenous ligand and is controlled by the concerted action of coactivator and corepressor proteins. The product of the thyroid hormone-regulated mammalian gene *hairless* (Hr) is a strong repressor of RORα transcriptional activity. In contrast to other corepressor-nuclear receptor interactions, Hr utilizes LxxLL motifs to mediate interaction with RORα, a mechanism associated with coactivator interaction. Strikingly, Hr specificity is dictated by the RORα AF-2 helix. Moreover, corepressor action is maintained in the presence of ligand, suggesting that Hr is a ligand-oblivious corepressor. The RORα AF-2 helix plays a

dynamic role in controlling both corepressor and coactivator interactions. The interaction of Hr with RORα provides a molecular link converging the thyroid hormone and RORα-mediated pathways in cerebellar development. RORα, like many nuclear receptors, is a short-lived protein whose turnover is mediated by the ubiquitin-proteasome pathway. Blocking of this pathway results in the accumulation of transcriptionally inactive ubiquitin-conjugated receptor. Both its endogenous ligand and coregulator binding perpetuate the degradation of this orphan. The ubiquitin-proteasome pathway plays an important role in regulating RORα-mediated transcription by concomitantly controlling protein stability and promoter occupancy, thereby inhibiting deleterious levels of transcription.

Résumé

Les récepteurs nucléaires sont des facteurs de transcription dont l'action est contrôlée par des petites molécules lipophiliques telles que les hormones stéroïdes, l'hormone thyroïdienne, et les vitamines A et D. Le récepteur nucléaire orphelin RORα a été cloné grâce à l'homologie qu'il partage avec le récepteur de l'acide rétinoïque. Le terme orphelin a été conçu pour décrire un récepteur dont la découverte a précédé celle de son ligand. L'inactivation du gène de souris *Rora* produit le phénotype «*staggerer*», caractérisé par une ataxie sévère du cervelet. RORα module l'activité de gènes impliqués dans la différentiation cellulaire, dont la myogenèse et l'adipogenèse, ainsi que dans diverses voies métaboliques. Les récepteurs nucléaires contrôlent la transcription de gènes cibles en se liant à de courtes séquences d'ADN, nommés éléments de réponse hormonales, à travers trois modes de liaison: monomère, homodimère ou hétérodimère avec RXR. La liaison de RORα à l'ADN est monomérique, car son domaine de liaison à l'ADN n'encode pas les déterminants moléculaires nécessaires pour la formation d'homodimères coopératifs. L'activité transcriptionelle du récepteur RORα dépend d'un ligand endogène, ainsi que de la liaison de corépresseurs et de coactivateurs. Le produit du gène mammifère *hairless* (Hr), un gène modulé par l'hormone thyroïdienne, réprime fortement l'activité transcriptionelle de RORα. L'interaction de Hr avec RORα se fait à travers des motifs LxxLL encodés par Hr, un mécanisme souvent utilisé par les coactivateurs et non par les corépresseurs. La répression par Hr se produit indépendamment de la présence de ligand, et l'interaction est dictée par la FA-2 du récepteur. La FA-2 contrôle l'interaction avec les corépresseurs et les coactivateurs et

joue un rôle dynamique dans l'activité transcriptionelle de RORα. Hr est donc le facteur commun qui relie les voies de signalisation menées par l'hormone thyroïdienne et RORα requises pour le développement du cervelet. RORα, comme d'autres récepteurs nucléaires, est une protéine instable qui est dégradée par le complexe de l'ubquitine-protéasome. L'inhibition du protéasome réprime la transcription modulée par RORα, dû a une accumulation marquée de récepteur inactif conjugué avec l'ubiquitine. La dégradation de RORα est initiée par les liaisons du ligand et des protéines corégulatrices. Le complexe de l'ubiquitine-protéasome module non seulement la stabilité de RORα, mais aussi sa durée d'occupation du promoteur de son gène cible afin d'éviter des niveaux de transcription nocifs à la cellule.

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This thesis is dedicated to my parents, Dionysios and Sofia, whose endless love and support helped me reach my goals. The completion of this thesis work pales in comparison to their achievements and hard work. A special thanks to my mother who is an amazing role model. To my brothers, Dimitri and Georges, thank you for your love and support. Finally, I would like to thank my fiancé Jimmy Katsipis for his love and for the happiness he has brought to my life. I especially thank him for his encouragement, and for all his support during the course of my thesis studies.

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Preface

The Guidelines Concerning Thesis Preparation Issued by the Faculty of Graduate Studies and Research at McGill University reads as follows:

> « The candidate has the option, subject to the approval of their department, of including as part of the thesis, the text of one or more papers submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to size, line spacing and margin sizes and must be bound together as an integral part of the thesis.
> The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.
> The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include, as separate chapters or sections: (a) a Table of Contents; (b) a general abstract in English and in French; (c) an introduction which clearly states the rationale and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary.
>
> As manuscripts for publication are very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
>
> In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's best interest to clearly specify the responsibilities of all the authors of the co-authored papers. »

I have chosen to write my thesis according to these guidelines, with two published papers, and one paper be submitted. The thesis is orpganized in six chapters: (I) a general introduction and literature review with references, (II-IV) manuscripts, each with its own abstract, introduction, methods, results, discussion and references, (V) a general discussion of all results with references, and (IV) contributions of authors.

Publications Arising from the Work of this Thesis

First-author Publications

1. Moraitis, A. N., and Giguère, V. (1999). Transition from monomeric to homodimeric DNA-binding by nuclear receptors: identification of RevErbAα determinants required for RORα homodimer complex formation, Mol Endocrinol *13*, 431-439.

2. Moraitis, A. N., Giguère, V., and Thompson, C. C. (2002). Novel mechanism of nuclear receptor corepressor interaction dictated by activation function 2 helix determinants, Mol Cell Biol *22*, 6831-41.

3. Moraitis, A. N., Giguère, V. Ubiquitin-Proteasomal Pathway Regulation of RORα Orphan Nuclear Receptor, *to be submitted.*

All figures in manuscripts 1, 2, and 3 were performed by A.N. Moraitis, with the exception of figure 3A of manuscript 2 which was perfomed by C.C. Thompson.

Other publication (not included in this thesis)

4. Sauvé, F., McBroom, L. D. B., Gallant, J., Moraitis, A. N., Labrie, F., and Giguère, V. (2001). CIA, a novel estrogen receptor coactivator with a bifunctional nuclear receptor interacting determinant, Mol Cell Biol *21*, 343-353.

In manuscript 4, figures 5 and 6 were performed by A.N. Moraitis.

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Chapter I Literature Review

1. Nuclear Receptors are Hormone-Inducible Transcription Factors

Hormones are chemical messengers critical to almost all aspects of mammalian physiology from development to regulation of cellular metabolism. They can control the intensity, timing and the direction of metabolic steps by binding to specific cell surface and intracellular proteins known as receptors. Nuclear receptors are DNA-binding transcription factors that transduce the effects of endocrine, paracrine, and intracrine hormones into orchestrated transcriptional responses crucial for embryonic development, homeostasis, fertility and many other biological processes. They interact with a chemically diverse array of ligands whose lipophilicity allows them to penetrate the plasma membrane and enter the cytoplasm and the nucleus (Figure 1).

In mammals, the nuclear receptor superfamily is composed of 48 genes that can be subdivided into two groups. The first group consists of the classical nuclear receptors, and encompasses receptors that bind steroid hormones, including estrogen (ER), progesterone (PR), androgen (AR), glucocorticoid (GR), and mineralocorticoid (MR), as well as thyroid hormone (T_3R), vitamin D (VDR) and vitamin A (RAR) (Table I). The steroid receptors are complexed to heat-shock proteins in the absence of their cognate ligands. Hormone binding leads to the dissociation of the heat-shock proteins, followed by homodimerization and translocation of the receptor into the nucleus, where it binds its hormone response element and regulates gene transcription (Figure 1) (reviewed in Tsai and O'Malley, 1994). The second group consists of nuclear receptors that have been termed 'orphans' given that the discovery of the re-

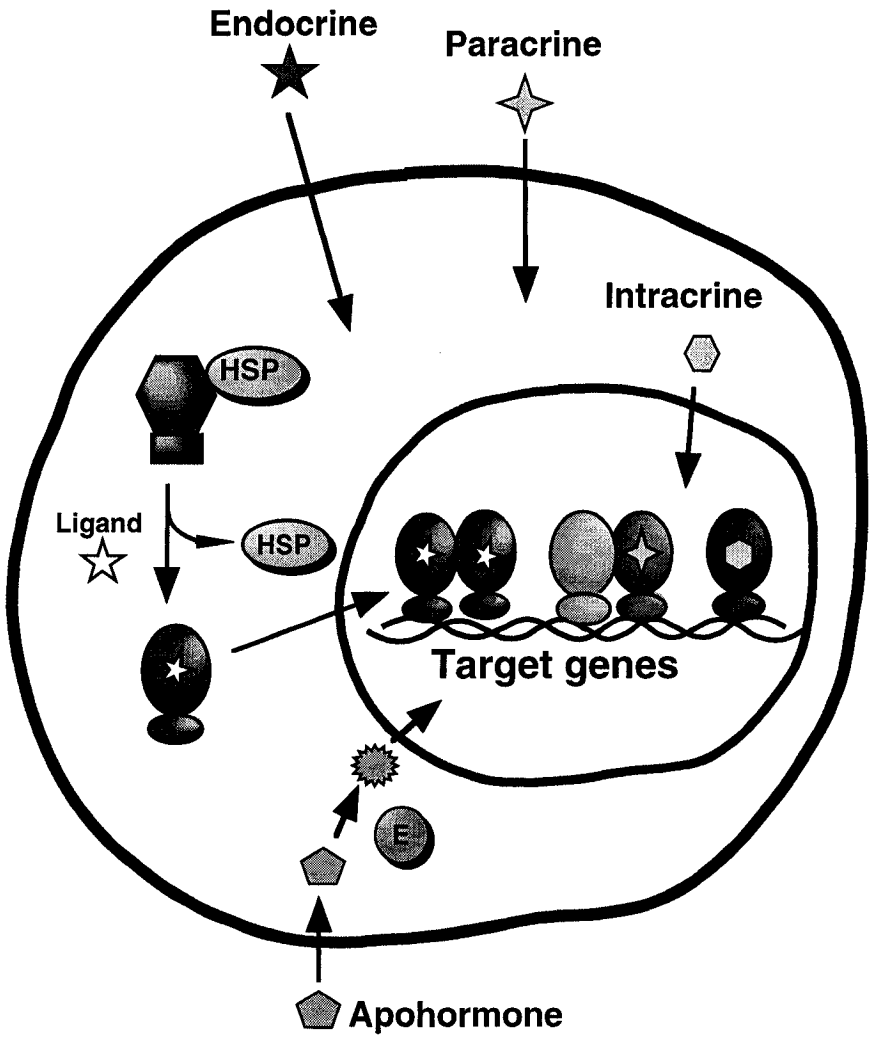


Figure 1. Hormonal signaling pathways regulate the genomic actions of nuclear receptors. Nuclear receptors are responsive to small lipophilic endocrine, paracrine, intracrine, or apohormones requiring enzymatic modification (E). Steroid receptors are complexed to heat shock proteins (HSP) in the cytoplasm. Upon ligand binding, they undergo dissociation and translocation into the nucleus. Nuclear receptors bind to DNA as homodimers, heterodimers or as monomers regulating the expression of their target genes.

ceptor has preceded that of the ligand (Giguère, 1999). A number of low affinity dietary ligands, derived from lipid metabolism or cholesterol derivatives, were subsequently identified as putative ligands for specific orphans receptors (Table I).

Nuclear receptors were named randomly as they were initially cloned from different species and by different groups, often resulting in a given receptor with various unrelated names. Most receptors are members of a subfamily of structurally related receptors, in which a Greek letter representing different genes identifies each isotype. For example, the RAR subfamily consists of RARα, RARβ, and RARγ isotypes, each encoded by a different gene. The multiple products of a given gene, due to alternative promoter usage or differential splicing, are isoforms of a given isotype and are represented by Arabic numerals, for example RARα1, RARα2, and RARα3. A unified nomenclature system was devised based on the system used for the cytochrome p450 superfamily (Nebert et al., 1987). In this system a gene name is given the prefix NR followed by an Arabic numeral for the subfamily, capital letters for the group, a second Arabic numeral for individual genes, and finally at the end a lower case letter is used for different isoforms, for example, RARα is designated as {NR1B1} (committee, 1999) (Table I).

2. A Historical Perspective

In the early 1900s, the field of endocrinology began with studies that demonstrated that thyroid extracts could control the metamorphosis of amphibians. In 1915, Kendall *et al* crystallized the molecule responsible for these effects and showed that it contained two iodinated tyrosine residues (Evans, 1988 and references therein).

Table I. The Nuclear Receptor Superfamily

Classic

Trivial Name	Nomenclature	High Affinity Ligand
GR	NR3C1	Glucocorticoids
MR	NR3C2	Aldosterone
PR	NR3C3	Progestins
AR	NR3C4	Androgens
ERα/β	NR3A1/A2	Estradiol
T_3Rα,β	NR1A1/A2	Thyroid hormone (TH)
VDR	NR1I1	1-25(OH)$_2$ vitamin D_3
RARα,β,γ	NR1B1/B2/B3	*all-trans* Retinoic Acid

Orphan

Trivial Name	Nomenclature	High Affinity Ligand
RXRα,β,γ	NR2B1/B2/B3	*9Cis*- Retinoic Acid
		Low Affinity Ligand
PPARα,β,γ	NR1C1/C2/C3	fatty acids, leukotriene β_4 prostaglandin J_2 thiazolidinediones
RevErbAα,β	NR1D1/D2	
RORα,β,γ	NR1F1/F2/F3	
LXRα,β	NR1H2/H3	oxysterols
FXR	NR1H4	bile acids
PXR	NR1I2	xenobiotics
CARα,β	NR1I3/I4	xenobiotics
HNF4α,β, γ	NR2A1/A2/A3	fatty acid
TR2α,β	NR2C1/C2	
TLX	NR2E1	
PNR	NR2E3	
COUP-TFα,β,γ	NR2F1/F2/F4	
Ear-2	NR2F6	
ERRα,β,γ	NR3B1/B2/B3	Diethylstilbesterol, tamoxifen
NGFI-Bα,β,γ	NR4A1/A2/A3	
SF1	NR5A1	oxysterols
LRH	NR5A2	
GCNF	NR6A1	
DAX1	NR0B1	
SHP	NR0B2	

In the 1930s, steroid hormones were discovered on the basis of their effects on development, differentiation, metamorphosis and physiological processes. The glucocorticoid hormone in adrenal gland extract was the first steroid hormone to be identified. Jensen *et al*, in 1966, introduced the use of radiolabeled hormone, and were the first to demonstrate that receptor proteins translocate from the cytoplasm to the nucleus. In 1985, Hollenberg *et al* cloned the first nuclear receptor, the glucocorticoid nuclear receptor (GR) {NR3C1}, which was also one of the first eukaryotic transcription factors cloned. This was closely followed by the cloning of the estrogen receptor (ERα) {NR3A1} (Green et al., 1986). The homology shared between GR and ERα, and the v-erb oncoprotein lead to the cloning of the c-erbA locus, which was later shown to encode the thyroid hormone receptor (TR) {NR1A} (Sap et al., 1986; Weinberger et al., 1986). In 1987, Giguère *et al.* and Petkovich *et al.* independently cloned the retinoic acid receptor (RAR), a protein transducing the effects of retinoic acid, a vitamin A metabolite. In 1988, the first insect nuclear receptor, the drosophila ecdysone receptor (EcR) was cloned, thereby suggesting that these hormone binding transcription factors evolved from a common primordial gene prior to the divergence of vertebrates and invertebrates (Nauber et al., 1988; Oro et al., 1988).

These studies established that nuclear receptors share two structurally related domains, the DNA binding domain (DBD) and the ligand binding domain (LBD), and have since served as the basis for the identification of additional members of a rapidly growing family. In 1988, using the ER DBD as a probe for homology screening, Giguère *et al* cloned the first orphan nuclear receptor, the estrogen-related orphan

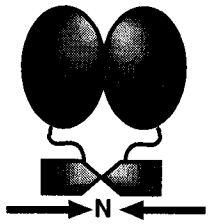
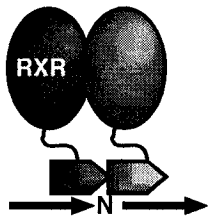
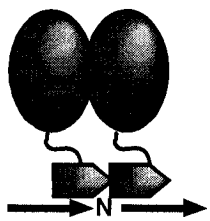
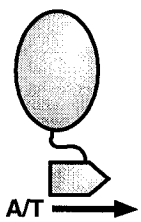
receptor (ERR) {NR3B}. Similar studies led to the identification of the chicken ovalbumin upstream promoter-transcription factor (COUP-TF) {NR2F} and the nerve growth factor-inducible (NGFI-B) {NR4A} orphan nuclear receptors (Milbrandt, 1988; Wang et al., 1989). Novel nuclear receptors were subsequently identified based on homology screening using genetic, biochemical and molecular biology techniques. A myriad of newly identified nuclear receptors launched the era of reverse endocrinology, where functional studies of the receptor protein precede the identification of the ligand. The first orphan ligand to be discovered was *9-cis* retinoic acid for the retinoid X receptor (RXR) {NR2B} (Mangelsdorf et al., 1990). Screening of selected sets of ligands, random screening of known drug molecules, and x-ray crystallography of the LBD of orphan receptors have been useful in identifying the ligands for approximately 20 orphan nuclear receptors. These 'orphan' ligands are low affinity lipophilic metabolites including fatty acids (PPAR) {NR1C}, bile acids (FXR) {NR1H4}, and oxysterols (LXR) {NR1H}, as well as xenobiotic molecules (PXR, CAR) {NR1I2, NR1I3} found in the environment. The ligands can provide important clues as to the physiological function of the receptor. The identification of ligands for orphan nuclear receptors has lead to the discovery of new hormonal pathways.

3. Hormone Response Elements

Nuclear receptors regulate gene transcription by binding to short DNA sequences termed hormone response elements (HRE) in the promoter region of their target genes. HREs are cis-acting and enhancer-like elements whose position and

orientation within the regulatory region of a given gene does not influence recognition by nuclear receptors. There are three modes of DNA binding by nuclear receptors resulting from receptor differences in the DNA binding domain and dimerization domains: monomeric, homodimeric and heterodimeric (discussed below) (Table II). HREs contain a minimal core hexamer consensus sequence, AGAACA (GR hexamer motif) that is recognized by steroid receptors. An exception is ER, which recognizes an AGGTCA motif (ER hexamer motif) that is also recognized by the rest of the nuclear receptor superfamily. This hexad core can be configured as a single half site flanked by a 5'-extension, favoring monomeric binding, or as tandem repeats that recruit nuclear receptor dimers. In a natural promoter, variations from the idealized sequence do exist and can be significant, often resulting in a lower binding affinity in comparison to a synthetic element. This may serve to attenuate the magnitude of the transcriptional response of a nuclear receptor in presence of ligand. Moreover, binding to natural nonconsensus binding sites has been shown to be dependent on cooperative interactions with adjacent transcription factors (Pearce and Yamamoto, 1993). For homodimeric or heterodimeric binding one consensus half-site is often sufficient to warrant high affinity binding. Inverted (palindromic) repeats (IR) spaced by 3 nucleotides of the AGAACA and AGGTCA motifs are bound by GR, MR, AR, or PR homodimers and ER homodimers, respectively. RAR, TR, and VDR, as well as a number of orphan nuclear receptors heterodimerize with the ubiquitous partner RXR, and bind direct repeats (DR) spaced by 1-5 bp, or inverted repeats spaced by 1 bp. There are three features of a HRE that regulate the specificity of DNA recognition and the three distinct modes of DNA

Table II. DNA Binding Modes

Binding Mode*	HRE	Classic	Orphan
Homodimer 	Inverted Repeat → AGAACA	GR, MR, PR, AR	
	AGGTCA	ERα,β	ERRα,β,γ
Heterodimer 	Direct Repeat → AGGTCA	T_3Rα,β; RARα,β,γ; VDR	PPARα,β,γ; LXRα,β; FXR; PXR; CARα,β; T_3Rα,β; COUP-TFα,β,γ; NGFI-Bα,β,γ
Homodimer 	Direct Repeat → AGGTCA		RXRα,β,γ; Rev-ErbAα,β; HNF4α,β,γ; TR2α,β; TLX; COUP-TFα,β,γ; NGFI-Bα,β,γ; Ear-2; GCNF
Monomer 	Half-site → AGGTCA		Rev-ErbAα,β; RORα,β,γ; ERRα,β,γ; NGFI-Bα,β,γ; SF-1; LRH; TLX; PNR

*inverted or direct repeats are spaced by 0-5 nucleotides (N), and single half sites are preceded by a 5'A/T rich flanking region (A/T).

binding: the specific sequence of the recognition motif itself, the orientation of the two half sites with respect to each other (inverted versus direct repeat), and the spacing between each core motif. The spacing between the two half sites selects for the given RXR heterodimer pair that will bind the HRE. For example, RXR-RAR heterodimer recognizes a DR5 (DR spaced by 5 nucleotides) whereas RXR-TR heterodimer preferentially binds to a DR4 element. In addition to spacing, specificity is also influenced by subtle differences in the hexameric core sequence as well as the 5'flanking sequence. Monomeric binding is a property that is exclusive to a small subset of orphan nuclear receptors. A single AGGTCA half site motif is usually preceded by an A/T-rich flanking sequence. In addition, a number of nuclear receptors recognize more than one type of HRE, therefore widening their range of potential target genes. For example, the ERR forms both homodimers and monomers binding to a IR3 and single half site, repectively (Johnston et al., 1997; Sem et al., 1997; Vanacker et al., 1999) (Table II).

4. Nuclear Receptor Anatomy

Nuclear receptors share four modular domains: the N-terminal or modulator domain (NTD), the DNA binding domain (DBD), the hinge region, and the ligand-binding domain (LBD), also designated A/B, C, D, E/F domains, respectively (Figure 2A) (Aranda and Pascual, 2001; Giguère, 1999; Kumar and Thompson, 1999; Owen and Zelent, 2000; Renaud and Moras, 2000). The modular character of these domains was initially demonstrated by mutagenesis and domain-swapping experiments (Giguère et al., 1986; Green and Chambon, 1987). There are two unusual receptors

Figure 2

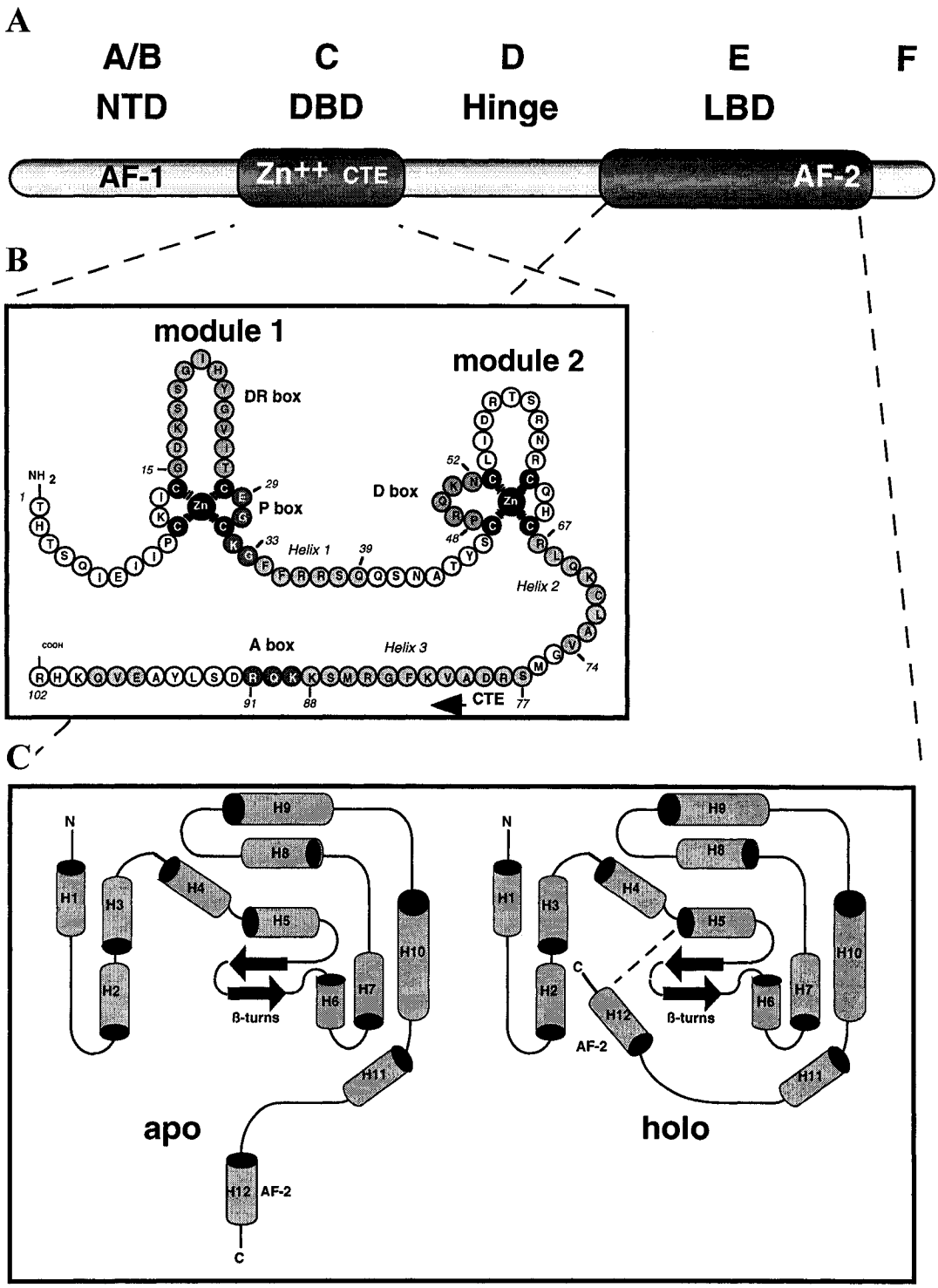

A
A/B
NTD
C
DBD
D
Hinge
E
LBD
F
AF-1
Zn++ CTE
AF-2
B
module 1
module 2
DR box
P box
D box
Helix 1
Helix 2
Helix 3
A box
CTE
NH 2
COOH
C
N
H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12
AF-2
ß-turns
apo
holo

Figure 2. Nuclear receptor anatomy. (A) Schematic representation of nuclear receptor modular domains: the A/B or N-terminal domain (NTD), the C or DNA-binding domain (DBD), the D or Hinge domain, the E or ligand-binding domain (LBD), and the F domain. The DBD consists of two zinc fingers (represented by ZN^{++}) followed by the C-terminal extension region (CTE). The NTD and LBD encode the activation function AF-1 and AF-2, respectively. (B) Schematic representation a typical nuclear receptor DBD. For simplicity the numbering begins at the first amino acid (aa) of the DBD. The two zinc finger modules tethering a zinc atom and the CTE (aa 77-102), as well as the three α-helices are identified. The DR box (aa 15-27) and the D box (aa 48-52) are involved in protein-DNA and protein-protein interactions. The P box (aa 29-33) provides DNA binding specificity. The CTE and the A box (aa 89-91) are involved in monomeric DNA binding. (C) The LBD is composed of 12 α-helices and 2 β-sheets folded into an anti-parallel sandwich. The secondary structure of the LBD in its apo (unliganded) and holo (liganded) conformation is represented, in the left and right panel respectively. The most striking change in conformation upon ligand binding is the position of the AF-2 helix (H12).

that share only the DBD or the LBD with the rest of the family, Dax-1 and SHP respectively.

The Modulator Domain. The NTD is a hypervariable domain that can vary both in length and primary sequence. Despite the lack of sequence conservation between different nuclear receptor family members, the NTD for a given receptor is well conserved through evolution. Receptor isoforms share identical DBDs and LBDs, but usually diverge in their NTDs (Chambon, 1996; Giguère, 1994). Very little is known about the structure of the NTD, except that it is generally rich in acidic amino acid residues and is unstructured in solution, based on circular dichroism and nuclear magnetic resonance studies (Dahlman-Wright et al., 1995). Results from treatment with trifluoroethanol (TFE), a strong α-helix stabilizer, suggest that the NTD may be composed of multiple α-helices (Dahlman-Wright et al., 1995; McEwan et al., 1993). The NTD is responsible for the transactivation function of nuclear receptors via a ligand-independent activation function (AF-1). The AF-1 acts in a cell and/or promoter context specific manner, suggesting that nuclear receptors may interact with cell specific cofactors. It has been demonstrated to interact with the Ada and TFIID complexes of basal transcriptional machinery via the Ada2 and TATA-binding protein, respectively (Ford et al., 1997; Henriksson et al., 1997). This domain also mediates interactions with coregulator, adaptor, and mediator proteins, as well as RNA molecules (Chakravarti et al., 1996; Kamei et al., 1996; Oñate et al., 1995; Zeiner and Gehring, 1995). The NTD is a target for post translational modification, namely phosphorylation by activation of signal transduction pathways (discussed

below). Moreover, it has been shown to modulate target gene specificity and influence DNA binding activity (Giguère et al., 1994).

The DBD, a hallmark domain. The hallmark domain that has defined the nuclear receptor superfamily is the DNA binding domain (DBD) (Glass, 1994). It is the most highly conserved domain, with 9 invariant cysteine residues, as well as a number of other invariant amino acid residues among nuclear receptor superfamily members. It has served as a fundamental tool in the identification of new family members. NMR spectroscopy and X-ray crystallization have been valuable means for determining the structure of the nuclear receptor DBDs free or complexed to DNA (Hard et al., 1990; Knegtel et al., 1993; Lee et al., 1993b; Luisi et al., 1991; Schwabe et al., 1993; Schwabe et al., 1990). The DBD encodes two class II zinc finger modules, of the $(Cys)_4$ type, where an amphipathic α-helix begins at the third cysteine of each zinc finger (Figure 2B). The zinc fingers were initially identified in the GR receptor and were compared with the zinc fingers of the *Xenopus* TFIIIA transcription factor (Brown et al., 1985; Miller et al., 1985; Severne et al., 1988). The classical zinc fingers of TFIIIA, and other DNA binding zinc finger proteins function as autonomous entities each independently contributing to DNA binding. In contrast, nuclear receptor zinc fingers modules fold together to form a compact interdependent structure that functions as a single globular domain, mediating both protein-DNA and protein-protein interactions (Baumann et al., 1993; Freedman, 1992; Hard et al., 1990). A third helix, involved in mediating appropriate DNA binding, is formed by amino acids extending past the second zinc finger module, and encompasses the carboxy-terminal extension (CTE) domain (Giguère et al., 1995b; Lee et al., 1993b).

Mutational analysis of the ER and GR DBDs revealed that helix 1 is the recognition helix involved in discriminating between the GR (AGAACA) and the ER (AGGTCA) consensus half-site motifs (Kumar et al., 1987). More specifically, the particular group of amino acids that are biased for a given consensus site lies between the last two cysteines at the base of the first zinc finger forming the P box (Danielsen et al., 1989; Luisi et al., 1991; Mader et al., 1989; Umesono and Evans, 1989) (Figure 2B). Moreover, amino acids flanking the P-box are involed in non-specific interactions with the DNA phosphodiester backbone stabilizing the association of the DBD with DNA. A change of three amino acids in the ER P-box confers the receptor with the ability to preferentially recognize the GR hexad sequence (Green et al., 1988; Umesono and Evans, 1989). The ER P-box is encoded by most receptors, and may be the ancestral P-box, mutation of which generated the GR P-box, restricted to adrenal and gonadal steroid receptors. In addition, amino acids localized to residues between the first two cysteines of the second zinc finger module forming the D box, are involved in discriminating for the optimal spacing between half sites (Figure 2B) (Umesono and Evans, 1989). Moreover, the D-box is also required for DNA-dependent protein-protein interactions between the DBD moieties of steroid homodimers (Umesono et al., 1991). A second dimerization interface is located in the LBD and is critical in mediating homodimerization in solution in absence of DNA.

The molecular determinants mediating RXR heterodimer formation are very distinct from those required for homodimer formation, mainly due to the asymmetric nature of the HREs that is bound. Unlike steroid receptors, which dimerize in a head-to-head fashion on an inverted repeat, RXR heterodimers bind to direct repeats,

palindromic or even inverted repeats, in a head-to-tail, head-to-head and even tail-to-tail fashion, respectively (Table II). This suggests that more than one interaction interface exists to accommodate these different binding conformations. Using base-specific cross-linking agents, various mutants and truncated forms of RXR, it was shown that a strict polarity exists on a DR element such that RXR always occupies the upstream recognition half site and its heterodimer partner the downstream site (Kurokawa et al., 1993; Predki et al., 1994). It is important to note that conformational flexibility exists given that on a DR1 element, RAR-RXR heterodimers are bound with the reverse polarity of other RXR heterodimers (Rastinejad et al., 2000). Similarly to steroid homodimers, RXR utilizes the D-box residues to mediate protein-protein interactions. On the other hand, given the inherent asymmetry of a direct repeat, the RXR partner employs residues within the first zinc finger module, termed the DR-box, to mediate dimerization as well as to dictate spacing specificity between core recognition motifs (Figure 2B) (Perlmann et al., 1993; Zechel et al., 1994).

The CTE, extending past the second zinc finger, was initially identified as a region essential for monomeric binding and stabilization owing to its involvement in making crucial contacts with the 5'A/T rich flanking sequence of a monomeric HRE (Table II) (Giguère et al., 1995b; Laudet and Adelmant, 1995). In addition to specifically distinguishing between 5'flanking sequences for monomeric binding receptors, the CTE also acts as a 'molecular ruler' discriminating against response elements with incorrect spacings, therefore contributing to DNA recognition by homodimer and heterodimer receptors. The crystal structures of RXR-T_3R and Rev-

ErbAα {NR1D1} DBDs showed that the CTE is also involved in both protein-DNA and protein-protein interactions, hence participating in both homodimerization and heterodimerization (Figure 2B) (Lee et al., 1993b; Rastinejad et al., 1995; Zhao et al., 1998).

The hinge domain, a flexible region. The hinge domain is not well conserved among members of the nuclear receptor superfamily, and varies greatly in length (Figure 2A). The hinge provides the receptor with the rotational flexibility necessary to pivot the LBD around the DBD, permitting heterodimerization on direct repeats, palindromic, and inverted repeats, each of which require different configurations (Glass, 1994). In conjunction with the NTD, it is also required for DNA bending, orienting the DBD and the CTE relative to each other in order to achieve proper protein-protein and protein-DNA interactions, as demonstrated with the ROR {NR1F} orphan nuclear receptor (McBroom et al., 1995). In addition, the hinge encodes a nuclear localization signal involved in shuttling nuclear receptors from the cytoplasm to the nucleus (Guiochon-Mantel et al., 1989). Moreover, this flexible domain also provides a docking site for heat shock proteins, as well as corepressor proteins (Carson-Jurica et al., 1989; Chen and Evans, 1995; Horlein et al., 1995). For the SF-1 {NR5A1} orphan receptor, the hinge domain has the additional function of participating in transcriptional activation due to the presence of an activation function domain, overlapping with a phosphorylation site (Desclozeaux et al., 2002; Li et al., 1999a).

The LBD, an Effector Domain. The LBD is the effector domain that transduces the signals mediated by the ligand, encodes a ligand-dependent activation function 2

(AF-2) domain, provides a platform for the binding of transcriptional mediators (transcriptional intermediary factors, coregulator proteins), forms a dimerization interface, interacts with heat shock proteins, and encodes a nuclear translocation signal. It is moderately conserved among family members allowing for distinct classes of lipophilic molecules to activate individual receptors (reviewed in Egea et al., 2000a; Escriva et al., 2000; Moras and Gronemeyer, 1998).

Initial biochemical assays demonstrated that upon ligand binding, nuclear receptors acquire an increased resistance to proteolytic digestion and an increased gel mobility suggesting that ligand binding induces a conformational change resulting in a more compact conformation of the liganded versus the unliganded receptor (Keidel et al., 1994; Leid, 1994; Leng et al., 1993). The crystal structure of a number of nuclear receptor LBDs both in absence (apo-) or presence (holo-) of their cognate ligands have been described, supporting this hypothesis; the dimeric apo-/holo-RXRα, monomeric holo-RARγ, monomeric holo-T_3Rα, dimeric agonist (estrogen)-/antagonist (raloxifene)-bound ERα, apo-/holo-PPARγ, holo-PR, and holo-VDR (Bourguet et al., 1995; Brzozowski et al., 1997; Egea et al., 2000b; Nolte et al., 1998; Renaud et al., 1995; Rochel et al., 2000; Tanebaum et al., 1998; Uppenberg et al., 1998; Wagner et al., 1995; Williams and Sigler, 1998). The LBD structure of a number of orphan nuclear receptors has also been resolved, including homodimeric ERRγ, monomeric RORβ, PPARγ/RXR heterodimer, and homodimeric HNF-4α (Dhe-Paganon et al., 2002; Gampe et al., 2000; Greschik et al., 2002; Stehlin et al., 2001). The tertiary structure of the LBD is conserved across the superfamily, consisting of a three layer, antiparallel sandwich of 11-12 α-helices (numbered H1-

H12) and a β-turn, at the very core of which is a hydrophobic ligand binding pocket (LBP) (Figure 2C) (Wurtz et al., 1996). Although all nuclear receptor LBDs have the same canonical fold, some variability does exist. The apo-LBD structure is in a very different conformation in comparison to the holo-LBD structure, the most striking difference being in the position of helix 12, encoding the core motif of the AF-2 (AF-2AD) also referred to as the AF-2 helix. The AF-2 helix is critical in the control of transcriptional activity, and is highly conserved among family members, consists of a ΦΦEΦΦ consensus motif (E being glutamic acid and Φ a hydrophobic amino acid).

The crystal structures of ligand bound-nuclear receptors revealed that the ligand is buried in the center of the hydrophobic ligand binding pocket (LBP), with no obvious accessible entry or exit points. The crystal structure of PPARγ LBD demonstrated that a ligand entry point may exist between H3 and the β-turn, allowing the ligand to access the LBP without disrupting the conformation of the whole LBD. The mouse-trap model, which describes an induced fit mechanism of ligand binding, may thus help explain how the ligand finds itself at the very core of the structure (Moras and Gronemeyer, 1998). The apo-position of the AF-2 helix unveils a passage through which the ligand can enter the hydrophobic core. The ligand likely evokes a conformational change, bringing about an induced fit and establishing contact with key residues within the vicinity. The ligand is lured in by the hydrophobic residues that line the pocket and is stabilized by a few polar residues at the bottom of the pocket. This entry is sealed in the holo-position by the AF-2 helix. The position of the amino acids involved in contacting the lipophilic molecule is conserved throughout the family when mapped out onto a linear representation. The anchoring polar

residues are also highly conserved among members of a given subfamily, suggesting that within each subfamily, the ligands are positioned in a similar manner. The shape of the LBP is inherently encoded by a given receptor to match its cognate ligand. The affinity of ligands for their respective nuclear receptors is generally quite strong where the dissociation constants are in the nanomolar to micromolar range. For example, the RAR LBP can accommodate two chemically distinct forms of retinoic acid (RA): *all-trans* RA and the *9-cis*-RA (Klaholz et al., 1998). Three residues diverge between RAR isotypes accounting for isotype-selective retinoids. In contrast, PPARγ has a much larger cavity accommodating binding of a multitude of ligands with different stereochemistries. However, increase of cavity volume and increase in the array of possible ligands is often at the expense of ligand binding affinity. The apo-conformation of the LBD is not necessarily the default state, given that the orphan nuclear receptor ERRγ was shown to be in a holo-position in the absence of ligand (Greschik et al., 2002).

Comparison of the unliganded apo-RXRα LBD structure with the *9cis*-RA-bound RXRα demonstrated the change brought about by the binding of the cognate ligand, enables the ligand-dependent AF-2 domain to become transcriptionally competent (Bourguet et al., 1995; Egea et al., 2000b). The ligand induces a repositioning the AF-2 helix from its apo-position, where it protrudes from the core of the LBD and is exposed to solvent, to its holo-position where it serves as a lid sealing the ligand binding cavity, thus compacting the structure of the LBD. The transconformation of the AF-2 helix leads to the positioning of key residues of the AF-2 AD core, along with residues from helices 3 and 4, forming a hydrophobic cleft exposed to solvent at

the surface of the LBD. This surface is favorable for coactivator protein interaction, yielding a transcriptionally active complex (Feng et al., 1998). Moreover, in some cases, the AF-2 and the AF-1 domain cooperate in regulating transcription (Leid et al., 1992). For many nuclear receptors, in the absence of ligand, the hydrophobic cleft is occupied by corepressor proteins, which evoke transcriptional silencing. The re-positioning of the AF-2 helix upon ligand binding disrupts this nuclear receptor-corepressor complex. Similarly, antagonist binding, as demonstrated by raloxifene-bound ERα, causes steric hindrance due to bulky side chains that block the AF-2 helix from assuming its transcriptionally active position, allowing the cleft to be occupied by corepressor proteins (Egea et al., 2000a). Ligand binding does not always translate to rearrangement of the LBD favoring coactivator binding. For the CARβ {NR1I4} orphan receptor, coactivator interaction is disrupted upon binding of the ligand, androstanol, which functions as a reverse agonist, silencing transcription (Forman et al., 1998). Similarly, binding of the ER agonist diethylstilbesterol or antagonist tamoxifen to ERR leads to inhibition of ERR activation due to ablation of coactivator interaction (Coward et al., 2001; Tremblay et al., 2001a; Tremblay et al., 2001b).

The LBD provides the primary interface required for dimerization in solution, high affinity DNA binding, as well as high degree of cooperativity between subunits. Deletion of the LBD, and consequently loss of this C-terminal dimerization interface, leads to a marked decrease in the DNA binding affinities of RAR and TR heterodimers (Glass et al., 1990; Perlman et al., 1992). The dimerization interface is formed by nine motifs, termed heptad motifs, initially predicted by mutagenesis studies to form α-helices involved in coiled-coil interactions (Forman et al., 1989). A

number of receptors were crystallized as homodimers or heterodimers, revealing that the key residues involved in dimerization lie in H10, and as postulated, have the ability to self-associate through hydrophobic contacts (Bourguet et al., 1995; Brzozowski et al., 1997; Gampe et al., 2000; Uppenberg et al., 1998). Additional residues that lie in helices H7, H9, and the loop connecting H7 and H8, also contribute to protein-protein contacts. In the absence of ligand the AF-2 helix is projected away from the very core of the LBD, in some cases docked onto the heterodimer partner. Interestingly, the AF-2 helix of one monomer is in close proximity to the other subunit, providing the basis for allosteric crosstalk between subunits. This could play a significant role in the function of a given nuclear receptor heterodimer and may account for the RXR subordination, that is unresponsive to its own ligand when complexed with RAR, TR or VDR receptors (Vivat et al., 1997). It may be naïve to imagine that nuclear receptors exist as single monomeric unliganded units while awaiting binding to a ligand or a dimer partner, given that it has been shown that unliganded RXR, in contrast to other apo-LBDs, oligomerizes forming tetramers which dissociate upon ligand binding. These tetramers may represent a "storage" form of RXR (Chen et al., 1998).

The Final Domain. The F domain, encompassing the amino acids extending past the AF-2 helix, is not conserved among members of the nuclear receptor superfamily, and is even absent in many nuclear receptors (Figure 2A). The length of this domain is conserved for steroid receptors, with the exception of the ER (Nichols et al., 1998). It has not been included in the crystal structures of the LBD published to date, and its function is not well understood. For steroid receptors, such as ER, it has been shown

to confer ligand specificity and influence transcriptional activity (Montano et al., 1995; Roux et al., 1996). The ER F domain modulates transcriptional activity by influencing the agonist/antagonist effectiveness of anti-estrogens (Montano et al., 1995), and was shown to be inhibitory to dimerization (Peters and Khan, 1999). The F domain, for some receptors, may also modulate interaction with coactivator proteins (Sladek et al., 1999).

5. Nuclear Receptor Regulation

Nuclear receptor-mediated transcription, in addition to being regulated by ligand binding, can be controlled by three mechanisms: interaction with coregulator proteins, covalent modification, and proteasomal degradation (Figure 3). Coregulators, namely corepressors and coactivators, are accessory proteins that are recruited to the nuclear receptor and in turn bring protein complexes to nuclear receptors essential for mediating transcription repression or activation, respectively (Glass and Rosenfeld, 2000; Horwitz et al., 1996; McKenna et al., 1999; Robyr et al., 2000). Coactivators can be defined as proteins that interact and potentiate the transcriptional activation functions of DNA-bound transcription factors (reviewed in Lee et al., 2001). Squelching on a given promoter hinted to the existence of coactivator proteins. Squelching consists of the reduced transactivation of a promoter regulated by a given nuclear receptor in presence of another independent activated receptor, not sharing the same response element. Biochemical and expression cloning have been useful tools for the identification of unbound or ligand bound nuclear receptor interacting proteins.

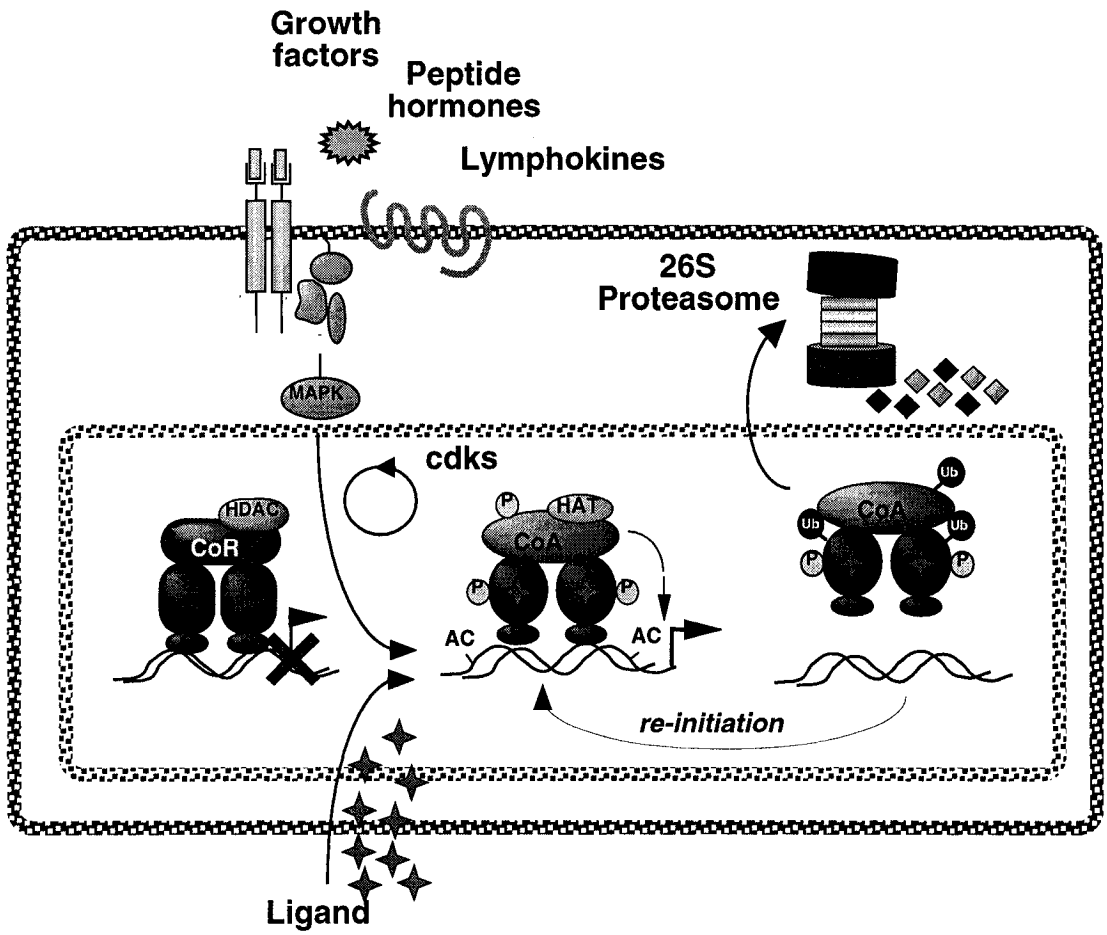


Figure 3. Regulation of nuclear receptor-mediated transcription. Nuclear receptors in absence of ligand interact with corepressors (CoR) often associated with HDACs leading to transcriptional repression. Ligand binding induces a conformational change favoring coactivator (CoA) binding, bringing HAT activity to the promoter region, leading to transcriptional activation. Nuclear receptor activation can also be regulated by phosphorylation of the receptor and/or coregulator, via signal transduction pathways elicited at the membrane by growth factors, peptide hormones, lymphokines, or by cell cycle kinases (cdks) during the cell cycle. The ubiquitin-proteasome pathway regulates transcription by degrading nucler receptors and coactivators, thus freeing the promoter for re-initiation of transcription.

Moreover, nuclear receptors can indirectly regulate transcription of a gene through protein-protein interactions with other transcription factors. Nuclear receptors are covalently modified, often involving phosphorylation by signal transduction pathways in response to events fired at the cellular membrane by growth factors, peptide hormones, lymphokines, or by cell cycle-dependent kinases. The cell has devised a mechanism to assure the proper level of transcriptional activation by proteolysis of nuclear receptors by the ubiquitin-proteasome complex in response to ligand stimulation, providing a fail-safe mechanism.

6. The Key Players of Transcriptional Repression

Repression plays a critical physiological role by regulating gene transcription through a number of different mechanisms. Competition for limiting coactivators in the nucleus can account for some aspects of gene repression. Passive repression involves competition for the same DNA binding element preventing binding of a strong activator, or quenching of an activator site by interference with activator function by binding to an overlapping site. Heterodimer formation can also serve as a repression mechanism given that it often yields a transcriptionally inactive complex. Alternatively, silencing transcription by active repression involves recruitment of the repressor directly to the DNA, creating an environment at the promoter that is incompatible with the assembly of the pre-initiation complex. Most unliganded receptors and antagonist-bound steroid receptors recruit corepressor proteins to their response elements, silencing the transcription of their target genes (reviewed in Hu and Lazar, 2000). In order for a protein to qualify as a *bone fide* corepressor it has to

possess an autonomous repression domain, interact directly with a nuclear receptor and induce basal transcriptional repression, often due to recruitment of histone deacetylases.

Histone Deacetylases Keep a Tight Grip on Chromatin. The basic structural unit of chromatin has been compared to "beads-on-a-string" conformation representing nucleosomes assembled onto DNA in repeating arrays. A nucleosome is composed of ~146bp of DNA wrapped two turns around an octamer containing two copies of histones H2A, H2B, H3 and H4. Histone H1 binds to the linker DNA between adjacent nucleosomes (reviewed in Kornberg, 1977; Kornberg and Lorch, 1992; Ramakrishnan, 1997). The core histones have N-terminal tails that extend from the octamer core in an unstructured manner. These tails contain lysine residues that can be reversibly acetylated on their ε-amino groups. When a gene is assembled into chromatin, its transcription is repressed due to the tight conformation of DNA wrapped around the histones making it inaccessible to transcription factors. Transcriptional repression correlates with hypoacetylation of histone N-terminal tails maintaining a repressive nucleosomal structure. Histone deacetylase enzymes (HDACs) deacetylate histone tails, favoring interactions between adjacent nucleosomes, which keeps the chromatin in a compact conformation and inhibits transcriptional activation (Luger et al., 1997). In humans, there are three highly homologous class I (HDAC1, HDAC2, HDAC3) and four class II (HDAC4, HDAC5, HDAC6, HDAC7) deacetylase enzymes. Class I HDACs form multisubunit complexes with mSin3A and the chromatin remodeling complex NuRD (nucleosome-remodeling histone deacetylase), and are involved in repression of T_3R and RAR by

promoting higher-order chromatin structure through deactelyation. Histone deacetylase activity is targeted to specific regions of the chromatin by interacting with sequence-specific DNA binding transcriptional repressors (reviewed in Ayer, 1999; Tong et al., 1998; Zhang et al., 1998c).

NcoR and SMRT are general corepressors. T_3R and RAR nuclear receptors in absence of their respective ligands are not simply transcriptionally inactive, like steroid receptors, they in fact repress the basal transcription of their target genes. A search for proteins that interact with unliganded T_3R and RAR led to the cloning of NCoR (Nuclear receptor Corepressor), a 270kD protein (Horlein et al., 1995; Kurokawa et al., 1995). Concurrently, a search for RXR-interacting proteins resulted in the cloning of RIP-13, encoding a segment of NCoR (Seol et al., 1996). A second 270kD RXR-interacting protein was purified by yeast two hybrid screen of a human lymphocyte cDNA library, and named SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor). This protein was also identified as TRAC2 (T_3R-Associated Cofactor 2), a protein isolated for its ability to interact with RXR, RAR and T_3R (Chen and Evans, 1995; Sande and Privalsky, 1996). NCoR and SMRT are structurally similar, both harboring multiple independent repression domains, and actively repress transcription when tethered to a heterologous DBD (Chen and Evans, 1995; Horlein et al., 1995). The N-terminal regions of NCoR and SMRT contain three repression domains (RD1, RD2, RD3) that do not share a high degree of homology amongst themselves, nor between respective NCoR/SMRT domains (Figure 4A). NCoR and SMRT RD1 and a region downstream of RD3 have been shown to interact with histone deacetylases HDAC1 and HDAC2 through direct interaction with

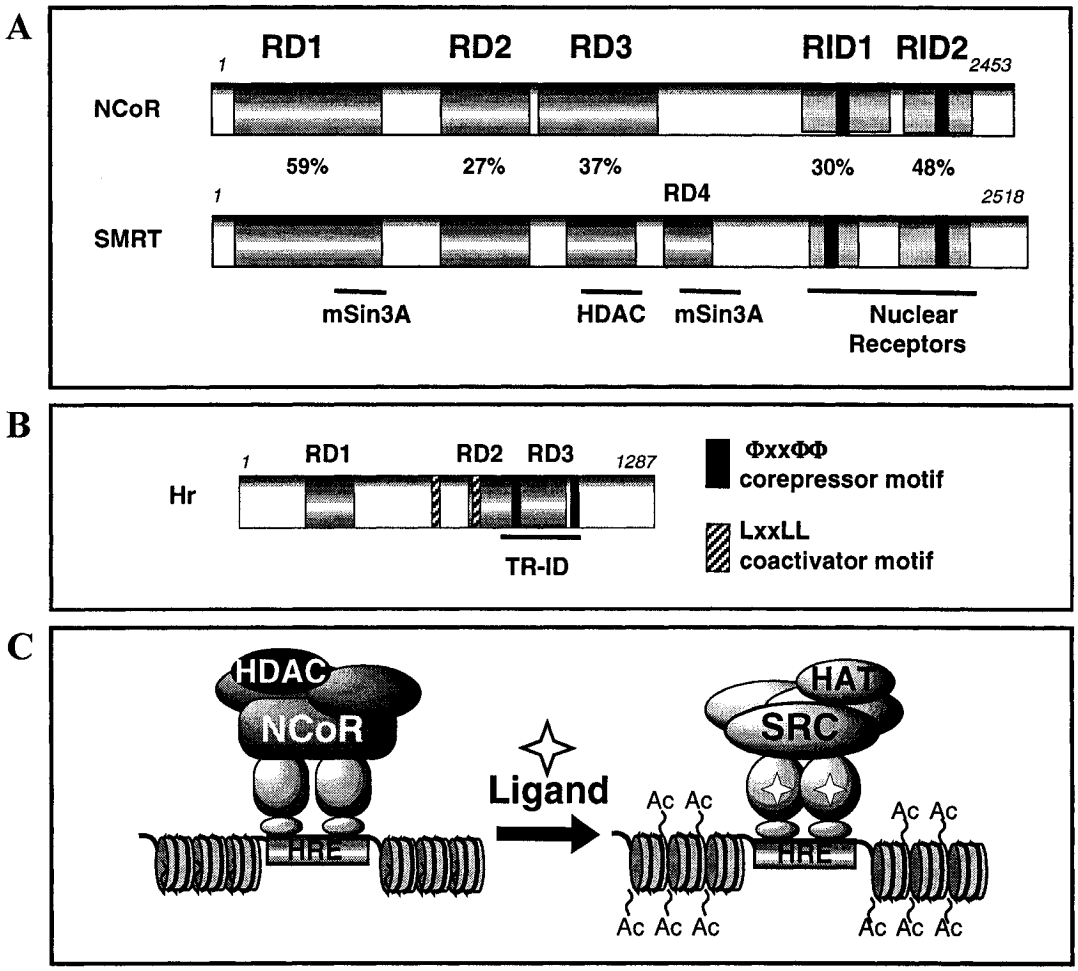


Figure 4. Corepressor Domain Structure. (A) Schematic representation of NCoR and SMRT corepressors. The percent homology shared between their repression domains (RD) and their receptor-interacting domains (RID) are indicated. Interaction domains for mSin3A, HDACs, and nuclear receptors are also indicated. (B) Hr corepressor encodes three RD and two ΦxxΦΦ corepressor motifs, forming the T_3R-interaction domain (TR-ID), and two LxxLL coactivator consensus motifs. (C) Model of coregulator exchange. Corepressor complex binding to nuclear receptors in the absence of ligand, silences transcription. Ligand binding induces binding of a coactivator complex containing HAT, acetylating histones (Ac) and resulting in activation of transcription.

mSin3A and mSin3B proteins, attributing to their ability to engage in the active repression of nuclear receptor signaling (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997; Wong and Privalsky, 1998b). Sin3 acts as a bridging molecule between the corepressor and the deacetylase complex. NCoR and SMRT can also interact with HDAC4, HDAC5, and HDAC7 (Huang et al., 2000; Kao et al., 2000). Purification of NCoR from *Xenopus* yielded three types of complexes: Sin3/HDAC1/RbAp48 complex, Sin3-independent containing HDAC complex, and an HDAC-free complex. Purification of NCoR from HeLa cells also resulted in the isolation of three types of complexes: the first contained HDAC1, HDAC2 and other components of the Sin3A-HDAC complex; the second included BRG-1, BAF170, BAF155, BAF47/INO1, Kap-1 (corepressor linked to heterochromatin silencing), and the third contained transducin β-like protein 1 (TBL-1), which shares structural and functional similarities to WD40-containing Tup1 and Groucho corepressors, and HDAC3. NcoR and SMRT have also been shown to interact with HDAC4 and HDAC5. Transcriptional silencing has been observed on chromatin free templates by T_3R suggesting that repression mechanism likely exists that are independent of histone deacetylases (Baniahmad et al., 1992; Damm et al., 1989; Sap et al., 1989).

The C-terminal domains of NCoR and SMRT harbor two receptor interacting domains (ID1 and ID2), each recruiting one partner of a heterodimer or homodimer pair (Figure 6A) (Cohen et al., 1998; Hu and Lazar, 1999; Perissi et al., 1999; Seol et al., 1996; Zamir et al., 1996; Zamir et al., 1997b). ID1 and ID2 have distinct affinities for specific receptors, and interact differentially with nuclear receptors. For example,

ID1 preferentially interacts with RAR and T_3R, whereas ID2 binds RXR (Wong and Privalsky, 1998c). Each ID encodes a NR box, referred to as the CoRNR box, encoding a conserved LxxI/HxxxI/L (L for leucine, I for isoleucine, H for histidine and x for any amino acid) or a minimal core ΦxxΦΦ (Φ being a hydrophobic residue and x any amino acid) sequence which forms an extended α-helix that anchors to the LBD of nuclear receptors (Figure 4A, C) (Nagy et al., 1999; Perissi et al., 1999).

NCoR and SMRT were originally isolated as factors interacting with the hinge region of unliganded T_3R and RAR. Helix 1 of the LBD encodes a small region termed CoR box initially thought to be required for direct interaction with NCoR or SMRT (Horlein et al., 1995). Mutation of this CoR box leads to a loss of both repression and interaction with corepressors, but more importantly also abolishes interaction with RXR, suggesting that residues encompassing this region are not directly involved in contacting NCoR or SMRT. Instead, corepressor determinants span residues in H3, H4 and H5 forming a hydrophobic cleft at the surface of the LBD, and corepressor binding is enhanced upon deletion of the AF-2 helix (Zhang et al., 1999). The position of the AF-2 helix, which is dictated by the ligand, influences the preferential binding of corepressors versus coactivators (Horlein et al., 1995; Kurokawa et al., 1995). Ligand binding induces a conformational change of the LBD where displacement of the AF-2 helix promotes corepressor dissociation. The length of the AF-2 helix and not the primary amino acid sequence is important in masking repression (Figure 4C).

Steroid receptors have very little DNA binding ability in the absence of ligand given that they are normally sequestered in the cytoplasm complexed to hsp90 or

hsp70. They are poor candidates for corepressor binding given that even if they bind DNA the position of their AF-2 helices in the apo conformation inhibits corepressor interaction. Although antagonist binding promotes DNA binding, it inhibits coactivator interaction and favors strong interactions with NcoR and SMRT, leading to transcriptional silencing of steroid receptor activity (Jackson et al., 1997; Lavinsky et al., 1998; Smith et al., 1997; Vegeto et al., 1992; Wagner et al., 1998; Zhang et al., 1998b). Interestingly, phosphorylation of the AF-1 domain by MAPK pathway allows ER and PR antagonists, tamoxifen and RU486 respectively, to switch from antagonists to agonists, recruiting coactivator proteins (Lavinsky et al., 1998). Although NCoR and SMRT share many of the same targets, they differ in their interaction with some orphan receptors. DAX-1, COUP-TF, and Rev-ErbAα orphan nuclear receptors interact quite strongly with NCoR, but interact very weakly if at all with SMRT (Crawford et al., 1998; Shibata et al., 1997; Zamir et al., 1996).

NcoR and SMRT function as corepressors not only to nuclear receptors but play a more general role in regulating transcription through interaction with numerous transcription factors. NCoR simultaneously interacts with TFIIB, $TAF_{II}32$, and $TAF_{II}70$ general transcription factors inhibiting the interaction of TFIIB and $TAF_{II}32$ required for transcriptional initiation (Muscat et al., 1998). NCoR is essential in the transcriptional repression mediated by Mad, a bHLH factor that forms a heterodimer with Max (Heinzel et al., 1997). SMRT interacts with the mammalian homologue of the *Drosophila suppressor of hairless*, CBF-1/RBP-JK, and is crucial to its ability to repress. NCoR/SMRT play an important role in homeodomain repressor function and interact with homeodomain factors Rpx and Pit-1, and mediate repression of

homeobox heterodimer pbx-hox (Saleh et al., 2000; Xu et al., 1998b). Other factors repressed by NCoR and SMRT are AP-1, NF- κB, SRF, MyoD, and E2F-repressive retinoblastoma protein (Bailey et al., 1999; Lai et al., 1999; Lee et al., 2000b).

The Role of NCoR and SMRT in human disease. NcoR and SMRT interaction with the POZ motif of the non-Hogkin's lymphoma-asociated protein LAZ3/BCL6, is of great physiological relevance in certain cases of acute promyelocytic leukemia, a disease characterized by incomplete leukocytic differentiation and the appearance of leukemic blast cells. Chromosomal translocations resulting in the fusion of RARα (DBD and LBD) with PML (promyelocytic leukemia) or the PLZF (promyelocytic leukemia zinc finger) both cause acute promyelocytic leukemia (APL) but have different clinical outcomes based on their interactions with NcoR and SMRT. Treatment with *all-trans* retinoic acid of patients with PML-RARα leads to remission, whereas there is no effect for patients with PLZF-RARα. The key difference at the molecular level lies in the interaction of these fusion proteins with corepressors. The PLZF motif constitutively recruits NCoR and SMRT irrespective of ligand binding to the RAR moiety, whereas recruitment by PML-RARα occurs through the RARα LBD which is reversed upon treatment with the ligand (Grignani et al., 1998; Hong et al., 1997; Lin et al., 1998; Wong and Privalsky, 1998a). Aberrant chromatin deacetylation may induce the leukemic state. Constitutive retention of corepressors by a nuclear receptor is implicated in many other clinical condition. For example, thyroid hormone-resistance syndromes can be correlated with T_3R LBD mutations that enhance ligand-independent interaction with NCoR/SMRT corepressors (Yoh et al., 1997). Regulation of both nuclear receptor and corepressor function is mediated

through signal transduction pathways. Post-translational modification of both receptors and corepressors alters their interaction. For example, activation of tyrosine kinases leads to a disruption of SMRT interaction with T_3R and the PLZF motif of the PLZF-RARα fusion, likely due to phosphorylation of SMRT (Hong et al., 1998). Corepressors are also regulated at the protein level in a cell-cycle dependent fashion or by proteasomal-mediated degradation, as observed with SMRT and NCoR-mSiah2 (mammalian seven in absentia homolog 2), respectively (Park et al., 1999; Zhang et al., 1998a). Genetic ablation of the mouse *NCoR* gene relieves nuclear receptor-mediated repression of specific genes resulting in a block of erythrocyte, thymocyte and neural development.

Nuclear receptor-specific Corepressors. There are a number of corepressor proteins identified that demonstrate nuclear receptor selectivity and share very little, if any, homology with NcoR and SMRT. Among these is TRUP (T_3R-uncoupling protein) which inhibits hormone-dependent transactivation and further enhances the silencing of unliganded T_3R and RAR by decreasing their ability to interact with their respective HREs (Burris et al., 1995). To date, TRUP has no effect on RXR or ER, or any other nuclear receptors tested. A small ubiquitous nuclear protein (~16kD), SUN-CoR encodes an intrinsic repression domain and interacts with both NCoR and SMRT (Zamir et al., 1997a). SUN-CoR enhances the silencing effect of T_3R and Rev-ErbA on basal transcription. Unlike NcoR and SMRT, it cannot be displaced from T_3R by thyroid hormone, and may be important for constitutive nuclear receptors (Zamir et al., 1996). Alien is a 41kD protein that functions as a corepressor for nuclear

receptors (Dressel et al., 1999). REA (repressor of ER activity) recognizes and represses antagonist-bound ER (Montano et al., 1999).

A mouse mutation induced by retroviral insertion resulting in the *hairless* phenotype was identified in 1926. Mapping of the insertion site and subsequent cloning of the gene led to the identification of the murine *Hairless* (*Hr*) gene (Brooke, 1926). The human ortholog is also associated with congenital hair disorders, namely *alopecia universalis* and *papular atrichia* (Ahmad et al., 1998; Ahmad et al., 1999; Cichon et al., 1998; Kruse et al., 1999; Sprecher et al., 1999). The precise biological role of Hr in hair growth remains to be elucidated. A search for thyroid hormone responsive genes in the rat cerebellum resulted in the cloning of the rat ortholog of the *Hr* gene (Cachon-Gonzalez et al., 1994; Thompson, 1996). This gene is rapidly induced by thyroid hormone and expressed in the rat cerebellum at high levels shortly after birth. It is a direct target gene of T_3R, encoding a T_3R response element in its promoter region. Moreover, Hr autoregulates its own expression by binding to unliganded T_3R, and repressing transcription (Thompson and Bottcher, 1997).

Hr is a nuclear receptor corepressor that specifically interacts with the T_3R LBD through two independent regions encoding hydrophobic motifs similar to those utilized by NCoR and SMRT (Figure 4B) (Potter et al., 2001). This T_3R-specific corepressor also localizes to subnuclear structures known as matrix-associated deacetylase bodies associated with SMRT and HDACs, suggesting that it is part of a large multisubunit complex. Similarly to NCoR and SMRT, Hr repression is mediated by recruitment of HDACs, specifically by interacting with HDAC1, 3 and 5. In contrast to the ubiquitous expression pattern and promiscuity of NCoR and SMRT,

Hr expression is restricted to the skin and brain and is nuclear receptor-selective. Moreover, the biological roles of Hr and T_3R are closely linked given that *Hr* mutant and TH-deficient animals share similar phenotypes. In the brain, morphological changes of Purkinje cells have been observed in both *Hr* mutant mice and TH-deficient mice (Garcia-Atares et al., 1998). In addition, hypothyroid mice exhibit cochlear defects that are also shared by Hr mutant mice (Cachon-Gonzalez et al., 1999; O'Malley et al., 1995). The shared phenotypic characteristics of these two mouse models underlines the importance of Hr-mediated repression of T_3R target genes.

7. Transcriptional Activation, a Complex Function

Transcriptional activity requires chromatin remodeling, involving translocation of the histone octamer relative to DNA catalyzed by the energy derived from ATP hydrolysis. This repositioning of the nucleosome likely involves breaking of the histone-DNA interactions and re-formation at adjacent nucleotides producing a DNA loop that is nucleosome-free and accessible by both specific transcription factors and general transcriptional initiation machinery. There are two types of chromatin remodeling factors: enzymes that hydrolyze ATP and enzymes that covalently modify nucleosomal histone proteins through acetylation.

Chromatin remodeling complexes, unwinding chromatin. The yeast SWI/SNF complex, yeast RSC (remodeling the structure of chromatin) complex, *Drosophila* NURF (nuclear remodeling factor), *Drosophila* CHRAC (chromatin-accessibility complex), Drosophila ACF (ATP-utilizing chromatin assembly and remodeling

factor), *Drosophila* BRM complex, and mammalian BRG1 or hbrm-associated complexes are remodeling enzymes that cause local changes in chromatin structure, leading to nucleosome disruption in an ATP-dependent manner, facilitating binding by transcription factors (reviewed in Grant et al., 1997; Hartzog and Winston, 1997; Ito et al., 1997; Kingston et al., 1996; Ogryzko et al., 1998; Pazin and Kadonaga, 1997a; Pazin and Kadonaga, 1997b; Peterson, 1996; Peterson and Tamkun, 1995; Struhl, 1999; Tsukiyama and Wu, 1995; Varga-Weisz et al., 1997; Wade and Wolffe, 1999; Wu, 1997). All these complexes contain a closely related ATP-binding subunit: SW12/SNF2 in yeast, ISW1 in *Drosophila* NURF, CHRAC, and ACF, and mammalian BRG1 or hbrm. The remodeling complex directly interacts with gene-specific transcription factors to ensure that chromatin remodeling is targeted to the right gene, creating a permissive chromatin environment for the assembly of the pre-initiation complex.

In general, the presence of nucleosomes around a promoter region can be inhibitory to transcription. Acetylation of the ε-amino group of lysine residues at the N-terminal of histone tails neutralizes their charge and weakens the electrostatic interaction between histones and DNA. This loosening of the chromatin structure renders the DNA more accessible to non-histone proteins such as transcription factors, and the basal transcriptional machinery. Alternatively, the histone tails may directly be involved in binding to transcriptional activators or chromatin-remodeling complexes (Georgel et al., 1997; Lee et al., 1993a; Vettese-Dadey et al., 1996). The degree of histone acetylation correlates with the rate of gene transcription. A link between histone acetylation and transcriptional activation was suggested upon

discovery that the *Tetrahymena* histone acetyltransferase A was related to the yeast transcription factor GCN5 (Brownell et al., 1996). Histone acetyltransferases (HATs) include: basal transcription factor TAF_{II250} subunit of TFIID, and transcriptional coactivators, GCN5 protein, CREB binding protein (CBP), the adenovirus E1A binding protein p300, p300/CBP-associated factor (P/CAF), and SRC family of coactivators (Bannister and Kouzarides, 1996; Brownell et al., 1996; Mizzen et al., 1996; Ogryzko et al., 1996; Spencer et al., 1997; Yang et al., 1996).

CBP, p300 and P/CAF cointegrators. CREB-binding protein (CBP) was initially characterized as a coactivator of the cAMP-response element-binding protein (CREB) by recruitment of RNA polymerase II to the promoter which is activated in response to cAMP (Kwok et al., 1994). p300 is homologous to CBP, and shares many functional similarities (Eckner et al., 1994). Both CBP and p300 function as coactivators for a number of nuclear receptors, and other transcription factors including p53, NF-κB, and MyoD (Avantaggiati et al., 1997; Chakravarti et al., 1996; Fronsdal et al., 1998; Kamei et al., 1996; Perkins et al., 1997; Yuan et al., 1996). Transcriptional enhancement of nuclear receptor activity is mediated through direct interaction via the N-terminal domain of CBP, which contains a NR-box encoding LxxLL consensus motifs (Kamei et al., 1996). In addition, CBP and p300 act as a scaffold, interacting synergistically with coactivators, enhancing nuclear receptor transactivation likely through the formation of a ternary complex. Although CBP and p300 share many similarities, they are not functionally redundant. Point mutation of the human *CBP* gene leads to the rare disorder Rubinstein-Taybi syndrome, characterized by severe mental retardation and physical deformities (Petrij

et al., 1995). Disruption of the murine *p300* gene leads to defects in neurulation, cell proliferation, heart development and embryonic lethality (Yao et al., 1998).

The p300/CBP-associated factor (P/CAF) is the mammalian ortholog of the yeast GCN5 transcription factor. GCN5 is a component of the yeast SAGA multisubunit coactivator complex, whose ability to activate transcription is dependent on the GCN5 HAT activity. The SAGA complex is composed of Ada proteins (Gcn5, Ada1, Ada2, Ada3, and Ada5), TATA-binding protein (TBP)-related Spt proteins (Spt3, Spt7, Spt8, Spt20), TAFs ($TAF_{II}90$, $TAF_{II}68/61$, $TAF_{II}60$, $TAF_{II}90$, $TAF_{II}25/23$, $TAF_{II}20/17$), and Tra1 (an ATM/PI-3 kinase-related homologue). Like its *Drosophila* counterpart, P/CAF is also the catalytic subunit of a large multisubunit complex, containing ADA family members, as well as TAFs, unable to acetylate histones autonomously (Ogryzko et al., 1998). P/CAF interacts with CBP/p300, SRC1, and SRC3 (described bbelow) (Chen et al., 1997; Spencer et al., 1997; Yang et al., 1996). In addition, it can also interact directly with RAR/RXR, ER, GR, AR nuclear receptors via their DBDs, potentiating ligand-mediated transactivation (Blanco et al., 1998).

The SRC coactivator family. The first nuclear receptor coactivator to be cloned was SRC-1 (Steroid Receptor Coactivator-1) based on its interaction with the PR LBD in a yeast two hybrid assay (Oñate et al., 1995). There are two functionally distinct isoforms of SRC-1, SRC1a and SRC1e, which differ in their C-termini (Kalkhoven et al., 1998). SRC-1 enhances ligand-dependent transcription for a large number of nuclear receptors in an AF-2 dependent fashion. SRC-1 has also been shown to enhance AF-1 mediated activity of the ER and AR nuclear receptors (Alen

et al., 1999; Bevan et al., 1999; Ma et al., 1999; Tremblay et al., 1999; Webb et al., 1998).

The search for nuclear receptor interacting proteins using various techniques led to the cloning of additional coactivators. A yeast two hybrid screen using the GR-LBD as bait resulted in the cloning of the GR-interacting protein 1 (GRIP1). Concurrently, a Far-western screen for ER and RAR-interacting proteins lead to the isolation of the transcriptional intermediary factor 2 (TIF2). Comparison of the mouse GRIP1 and human TIF2 revealed that a high homology is shared between these orthologs. GRIP1/TIF2 also share significant homology with SRC-1, establishing the existence of the SRC family of coactivators, and given the designation of SRC-2 (reviewed in Leo and Chen, 2000). The third member of this family was reported simultaneously by several groups as a CBP-interacting protein (p/CIP), RAR-interacting protein (RAC3), a gene amplified in breast cancer (AIB-1), a hRARβ-stimulatory protein (ACTR) and as T_3R-interacting protein (TRAM-1) or SRC-3/pCIP/RAC3/ACTR/TRAM-1/NcoA3 (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997; Takeshita et al., 1997; Torchia et al., 1997). Coactivation by SRC is not limited to members of the nuclear receptor superfamily given that SRC-1 enhances the activities of NF-κB, SMAD3, and AP-1 transcription factors, while pCIP enhances the activity of interferon-α and CREB (Lee et al., 1998; Na et al., 1998; Torchia et al., 1997; Yanagisawa et al., 1999).

The members of the SRC family share common structural domains (Figure 5). The most highly conserved domain is the bHLH (basic Helix-Loop-Helix) and PAS (Per/Arnt/Sim) domain located at the N-terminus (Figure 5). The bHLH domain is

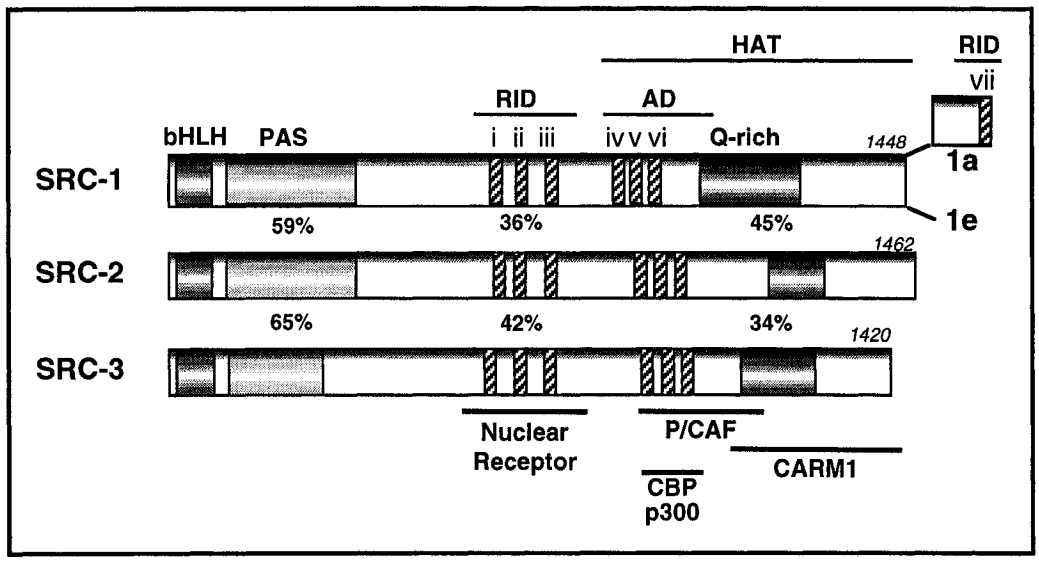


Figure 5. SRC family domain structure. The three members of the SRC family encode: a basic helix-loop-helix domain (bHLH), a Per/Arnt/Sim homologous domain (PAS), a receptor-interacting domain (RID) containing three NR boxes (i, ii, iii) each encoding a consensus LxxLL coactivator motifs, an activation domain (AD) also containing three NR boxes (iv, v, vi), a HAT domain, and a glutamine-rich region (Q-rich). There are two isoforms of SRC-1, SRC1a and SRC1e, SRC1a encodes an additional NR box (vii). The percentage homology shared between SRC-1, SRC-2 and SRC-3 with respect to their bHLH/PAS, RID and Q-rich domains is indicated. Regions essential for mediating interaction with nuclear recptors, P/CAF, CBP/p300 and CARM1 are also indicated.

also found in other transcription factors, such as the MyoD family in which it functions as a DNA binding domain or dimerization surface. Period (Per), Aryl hydrocarbon receptor (AhR), and the single-minded (Sim), transcriptional regulators encode a similar PAS domain shown to be involved in protein-protein interactions. Despite the clear role of these domains in other proteins, the function of this highly conserved domain in SRC proteins remains unknown, although it is hypothesized that it may be involved in intra- or intermolecular interactions.

The central region of SRC family members encodes the receptor-interacting domain (RID), followed at the C-terminus with an activation domain (AD) and a glutamate-rich region. The RID mediates interaction of SRC members with ligand-activated nuclear receptors (Li and Chen, 1998; Oñate et al., 1998; Voegel et al., 1998). The hallmark of this domain is the RID (receptor-interacting domain) encoding three conserved signature LxxLL motifs (where L is a leucine residue and x is any amino acid), also referred to as NR boxes, which contact the liganded nuclear receptor (Ding et al., 1998; Heery et al., 1997; Torchia et al., 1997). The SRC-1a isoform contains an additional LxxLL motif at the very C-terminal end of the protein. The LxxLL motif forms an amphipathic α-helix that anchors onto the hydrophobic cleft, formed by residues of helices 3, 4, 5, and the AF-2 helix, at the surface of the nuclear receptor LBD. Ligand binding leads to restructuring of the LBD such that the highly conserved glutamic acid residue of the AF-2 helix and the lysine residue of helix 3 form a charged clamp locking the LxxLL helix into place (Darimont et al., 1998; Feng et al., 1998; Nolte et al., 1998; Shiau et al., 1998; Torchia et al., 1997). This model was further supported by the crystal structure of the agonist bound ERα-

LBD complexed to SRC-2 NR box peptide. Antagonist binding shifts the AF-2 helix into a position that occludes the coactivator-binding site. Therefore, the transcriptional inactivity of the antagonist-bound receptor is coupled to its inability to interact with SRC coactivators. The crystal structures of the holo-$T_3R\beta$ LBD bound to SRC-2, and holo-PPARγLBD bound to a SRC-1 fragments encoding NR boxes i and ii, demonstrated that a single LxxLL peptide interacts with each monomer of the $T_3R\beta$ and PPARγ dimer, respectively (Darimont et al., 1998; Nolte et al., 1998). In addition, nuclear receptors encode an intrinsic LxxLL-like motif (LxxML) in the AF-2 helix which may mimic the coactivator signature peptide by contacting residues in the hydrophobic cleft, providing an explanation for the allosteric inhibition of the RXR-RAR heterodimer by the RXR AF-2 (Westin et al., 1998).

Despite the ability of a given SRC protein to interact with a number of nuclear receptors using three putative NR-boxes, a specific nuclear receptor-NR box code does exist, where different receptors preferentially interact with specific NR boxes of the RID (Darimont et al., 1998; Ding et al., 1998; Leers et al., 1998; McInerney et al., 1998). Most nuclear receptors require two intact NR boxes for interaction, whereas the mutation of a single LxxLL motif does not abolish this interaction. NR specificity is also provided by the spacing between NR boxes, and by the residues flanking the LxxLL motif (Chang et al., 1999; Darimont et al., 1998; McInerney et al., 1998). Particularly the eight amino acids C-terminal to this motif were shown to be essential for the coactivation by SRC-1 of RAR, T_3R, and ER (McInerney et al., 1998). The C-terminal domain of SRC family members may also be required for an interaction with a given nuclear receptor via the AF-1 domain, resulting in the enhancement of ligand-

independent transactivation, as demonstrated for AR (Alen et al., 1999; Bevan et al., 1999). Complete coactivation of AR requires synergism between both the AF-1 and AF-2 domains with members of the SRC family. ER AF-1 activity is enhanced by recruitment of SRC coactivators both in the presence of estradiol or tamoxifen. The partial agonism displayed by tamoxifen likely occurs through the AF-1 domain (Webb et al., 1998). Interaction with the SRC coactivator via AF-1 has been observed for a number of receptors (Lavinsky et al., 1998; Oñate et al., 1998; Tremblay et al., 1999; Webb et al., 1998).

SRC family members encode intrinsic transcriptional activation domains (AD) at their C-terminal ends that are responsible for their transcriptional activity, which is manifested when tethered to DNA by a heterologous DBD. AD1 contains three consensus LxxLL motifs, which unlike those of the RID, are required for interaction with CBP/p300 cointegrator proteins containing histone acetylase activity (HAT). CBP/p300 is indispensable for the coactivation function of SRCs via RAR (McInerney et al., 1998). SRC can also mediate activation in a CBP-independent manner via its AD2 domain (Ikeda et al., 1999; Ma et al., 1999; Voegel et al., 1998).

SRC-1 and SRC-3 also contain weak intrinsic HAT activity and may contribute to chromatin remodeling. Acetylation is an integral part of nuclear receptor activation, given that both CBP/p300 and SRC can be directly recruited by the receptor. Recruitment of HAT-containing cofactors to the promoter by nuclear receptors may modify the chromatin structure such that the access to the promoter is facilitated for the DRIP/TRAP mediator complex (described below), or the assembly of the pre-initiation complex. CBP/p300 and SRC-1 have been shown to interact with TBP and

TFIIB (Ikeda et al., 1999; Kwok et al., 1994; Takeshita et al., 1996; Yuan et al., 1996). The recruitment of numerous HATs may also be required for acetylation of non-histone proteins. Basal transcription factors TFIIE and TFIIF are acetylated by CBP and P/CAF *in vitro*, and transcription factors may also be targets for acetylation as seen with p53 (Gu and Roeder, 1997). Coactivator proteins themselves may be potential targets as well, given that SRC-3 has been shown to be a CBP/p300 substrate, leading to dissociation of SRC-3 from DNA-bound ER homodimer (Chen et al., 1999b).

There is some redundancy within the SRC family in terms of interaction and enhancement of nuclear receptor transcriptional activity, but these activities may not overlap *in vivo*. The SRC-1 knock-out mouse is viable, due in part to the compensatory role of overexpressed SRC-2 in certain tissues, although the levels of SRC-3 are unchanged (Xu et al., 1998a). Another example supporting the different functional roles of SRC family members is in breast cancer cells which display overexpression of SRC-3 whereas expression of SRC-1 or SRC-2 are relatively low (Anzick et al., 1997).

Other coactivators and components of the activation complex. In addition to interacting with nuclear receptors and cointegrators, SRC family members also recruit other coactivators, methyltransferases and RNA molecules to the activation complex. SRC family members recruit the coactivator-associated arginine methyltransferase I (CARM1) protein. CARMI, homologous to PRMT (protein methyltransferase), was cloned by yeast two hybrid assay using the C-terminus of GRIP1 (aa 1121-1462) as bait (Chen et al., 1999a). Interaction with all three family members is mediated

through the CBP/p300-independent activation domain, AD2 (Ma et al., 1999). CARM1 has a strong histone methyltransferase activity, with specific preference for histone H3. It enhances the transcriptional activity of SRC-2 tethered to the GAL4 DBD, and further potentiates SRC-2-mediated coactivation of AR, ER and T_3R, but does not directly enhance the transcriptional activities of nuclear receptors in absence of SRCs. While the AD domains of SRC each recruit proteins with different enzymatic activities, where AD1 is needed for CBP/p300 interaction and histone acetylation activity, and AD2 for CARM1 interaction and histone methylation, both contribute to chromatin remodeling. An effort to clone AF-1 specific coactivators lead to the cloning of the steroid receptor RNA (SRA), by yeast two-hybrid screen using the PR AF-1 domain as bait (Lanz et al., 1999). Surprisingly, SRA is an RNA transcript and not a protein that enhances steroid receptor (PR, GR, ER, AR) activity only. SRA may also function as a ribonucleoporetin scaffold through which the SRC complex enhances AF-1 mediated transactivation.

Unlike the SRC coactivators that are ubiquitously expressed, some coactivators are cell type and/or promoter-specific. The PGC-1 (PPAR gamma coactivator-1) coactivator was initially identified as a PPARγ specific coactivator, however it is now clear that this coactivator has a much broader repertoire of nuclear receptor targets, including GR, MR, ER, T_3R, RXR, and ERRα and γ (Delerive et al., 2002b; Huss et al., 2002; Knutti et al., 2000; Puigserver et al., 1998; Tcherepanova et al., 2000; Wu et al., 2002). PGC-1 also interacts with SRC-1 and p300 (Puigserver et al., 1999). It is preferentially expressed in tissues that have a high energy demand, and are rich in mitochondria, such as brown fat, skeletal muscle, heart kidney, liver and brain.

Interestingly, expression of this coactivator is induced upon exposure to cold temperatures in brown fat and muscle. Similarly, in fasting conditions or, during exercise, it is upregulated in the heart and muscle, respectively. PGC-1 is therefore involved in adaptive thermogenesis and regulation of energy metabolism. This metabolic coactivator induces mitochondrial biogenesis, and regulates the mitochondrial energy metabolism required to meet the cellular needs related to energy metabolism (reviewed in Knutti and Kralli, 2001).

A search for coregulator proteins that function independently of the AF-2 domain, using RVR (Rev-ErbAβ) orphan nuclear receptors which are devoid of an AF-2 helix, led to the isolation and cloning of a coactivator protein termed coactivator independent of AF-2 function (CIA) (Sauvé et al., 2001). Interestingly, CIA possesses both intrinsic activator and repressor functions, as well as a bifunctional NR recognition helix encoding overlapping coactivator LxxLL and corepressor ΦxxΦΦ core motifs. CIA interacts with both Rev-ErbAα and RVR orphan nuclear receptors, but is unable to either potentiate their repressive activity or activate Rev-ErbAα/RVR-mediated transcription. Despite the lack of binding to a number of steroid and nonsteroid nuclear receptors, CIA specifically binds ERα and ERβ in an AF-2 independent fashion, and enhances ERα estrogen-dependent transcriptional activity. Strikingly, CIA displays antiestrogen selectivity by interacting with EM-652- and ICI182,780-bound ERα but not when the receptor is bound to 4-hydroxytamoxifen or raloxifene. CIA represents a novel type of coregulator protein with properties attributed to both coactivator and corepressor proteins (Sauvé et al., 2001).

The Mediator Complex. The basal transcriptional machinery is composed of general transcription factors, RNA polymerase II (RNAP II) and the Mediator complex (reviewed in Rachez and Freedman, 2001; Woychik and Hampsey, 2002). The yeast mediator complex, comprising 20 subunits, is essential for transcriptional activation and has been shown to physically interact with RNAP II. It regulates the activity of the TFIIH C-terminal domain (CTD) kinase that phosphorylates the CTD of RNAP II, signaling the end of transcriptional initiation, and the launching of the enzyme into the elongation process. A large number of proteins isolated in either the RNAP II holoenzyme complex or the Mediator complex overlap but one complex is not necessarily a stoichiometric component of the other. In addition, the Mediator complex also acts as a coactivator independently of its association with RNAP II. In human, there are multiple Mediator complexes and their compositions vary in concordance with different strategies employed for isolation and based on interaction with various transcriptional activators. The search for cofactors that interact with ligand bound $T_3R\alpha$ nuclear receptor led to the identification of the TRAP (T_3R-associated proteins) complex, a multiprotein complex sharing similarities with the yeast mediator complex. This TRAP complex coactivates ligand mediated transcription of T_3R on naked, chromatin-free DNA (Fondell et al., 1996). Along parallel lines, a highly homologous complex composed of DRIPs (VDR interacting proteins) was purified using liganded VDR as bait (Rachez et al., 1998). DRIP potentiates ligand-mediated transactivation of VDR specifically on a chromatinized template, suggesting a potential unidentified chromatin remodeling function (Rachez et al., 1999). The TRAP/DRIP multiprotein complex embodies at least 9 proteins

ranging from 70 to 230kD, quite distinct from other coactivator complexes, and is devoid of HAT activity (Rachez et al., 1999; Yuan et al., 1998). Other multisubunit complexes were also identified including the ARC, human mediator, and the NAT/SMCC complexes based on their interaction with SREBP-1a/NF-κB/VP16, E1A and Srb transcriptional activators, respectively. These complexes range in size and composition but all contain proteins homologous to components of the yeast mediator complex. Different activator-specific human mediator complexes likely exist in the nucleus acting as a bridge between RNAP II and DNA-bound activators. For example, the DRIP complex interaction with RNAP II requires the presence of liganded-VDR, and does not interact with the polymerase in absence of a DNA-bound receptor.

A 220kD member of this TRAP/DRIP mediator complex, TRAP220/DRIP205 is identical to the PPARγ-binding protein, PBP (Zhu et al., 1997). This DRIP205/TRAP220/PBP subunit mediates the interaction with non-steroid nuclear receptors, in an AF-2 dependent fashion, through two alternatively utilized coactivator consensus LxxLL motifs, anchoring the rest of the complex to the receptor (Font de Mora and Brown, 2000; Kato, 2001; Rachez et al., 1999; Yuan et al., 1998). Disruption of the murine *DRIP205/TRAP220/PBP* gene leads to embryonic lethality, suggesting that it is indispensable for proper cellular function (Ito et al., 2000; Zhu et al., 2000).

Transcriptional Initiation by Complex Recruitment. Transcriptional initiation by nuclear receptors is a multistep process involving a large number of proteins with various functions and enzymatic activities (as described above) and the recruitment of

RNAP II and general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF AND TFIIH. The first step requires derepression of transcription by loosening of the chromatin structure via nucleosome disruption, in an ATP-dependent manner, which is mediated by chromatin remodeling complexes such as the SWI/SNF complex. Further nucleosome modification is executed by HAT-containing proteins, which acetylate histone tails and weaken histone interaction with DNA. This allows for recruitment of a coactivator complex by the DNA-bound nuclear receptor (Figure 6). This second complex brought to the promoter consists of cointegrators CBP/p300, and SRC coactivators, both containing HAT activity and promotes further unwinding of the chromatin structure, allowing for recruitment of basal transcriptional machinery. The third complex that comes into play is the Mediator complex that serves as a link between nuclear receptors and the RNAP II holoenzyme, and the subsequent assembly of the GTFs forming the pre-initiation complex. Following transcriptional initiation, the RNAP II CTD is transiently phosphorylated, signaling the commencement of transcriptional elongation. It has been suggested that the Mediator complex remains at the promoter, with a subset of GTFs (TFIID, TFIIA, TFIIH, TFIIE), forming a scaffold permitting reassembly of RNAP II, TFIIF and TFIIB for the re-initiation of transcription (Yudkovsky et al., 2000).

The exact sequence in which these different complexes are recruited to the promoter region by nuclear receptors is open for speculation. Given, that both the Coactivator and the Mediator complexes recognize an overlapping domain using the same type of recognition motif, namely LxxLL motifs, both complexes cannot be recruited to a receptor simultaneously. Three mechanisms have been proposed for the

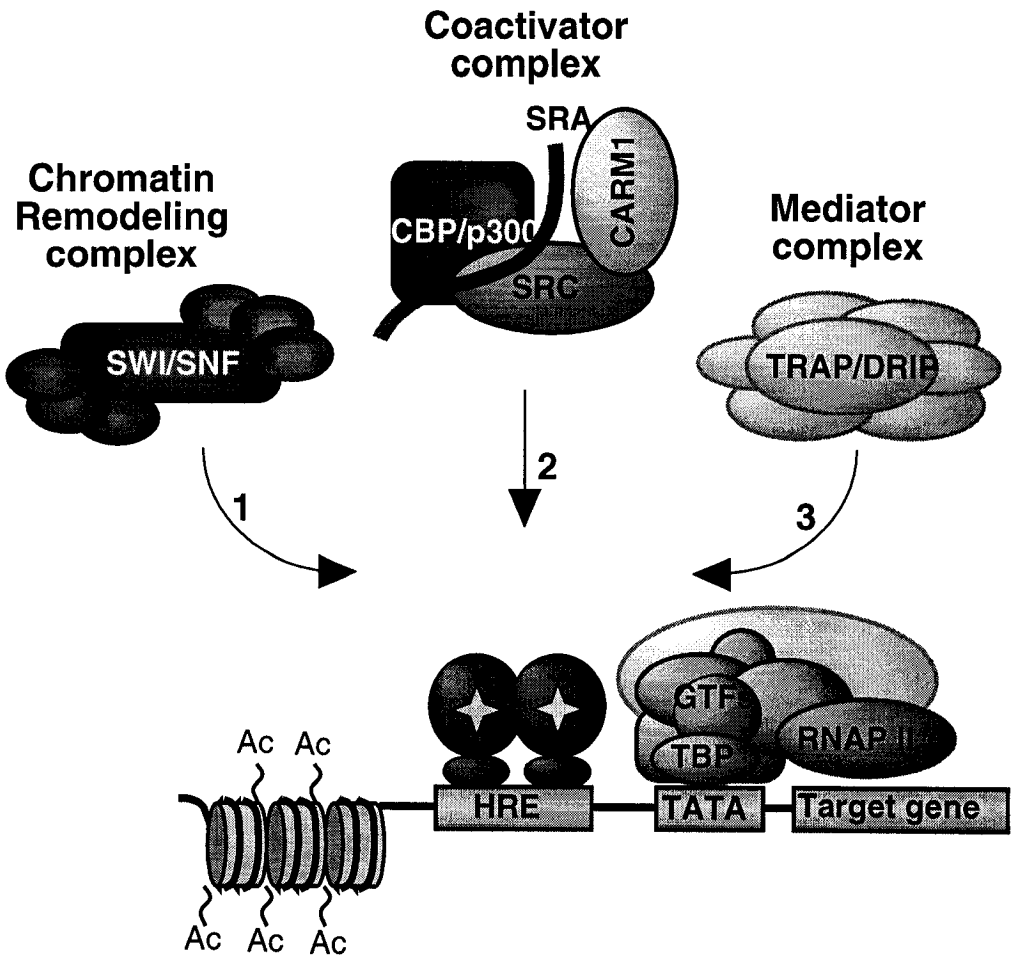


Figure 6. Coregulator exchange induced by ligand binding. Three different complexes are recruited by nuclear receptors for transcriptional activation: (1) the Chromatin remodeling complex (Swi/Snf) required for unwinding of the chromatin; (2) the Coactivator complex containing SRC coactivators, SRA, as well as CBP/p300, and CARM1 bringing acetyltransferase and methyltransferase activities respectively, to the promoter; (3) the Mediator complex (TRAP/DRIP), favoring the formation of the pre-initiation complex, and recruitment of the basal transcription machinery to the promoter, composed of general transcription factors (GTFs) and the RNA polymerase II (RNA Pol II).

exchange or recruitment of the three major activation complexes by nuclear receptors (Glass and Rosenfeld, 2000). The first possibility is a sequential mechanism in which initial recruitment of chromatin remodeling factors is followed by an exchange for the coactivator complex that is then replaced by the Mediator complex allowing for transcription to begin. The second plausible mechanism has been described as a combinatorial process, where synergy occurs between the Coactivator and the Mediator complex upon simultaneous recruitment by each subunit of a nuclear receptor dimer or by a set of dimers bound to a promoter encoding multiple HREs. Alternatively, this mechanism may also describe the recruitment of a nuclear receptor-specific coactivator on one set of promoters and the Coactivator/Mediator complex on another. The third possibility that can be envisaged is a parallel mechanism where a given gene is activated independently by different coactivator complexes (Figure 7).

8. Signal Transduction Meets Transcription

Nuclear receptors integrate signals both from their ligands as well as those emanating from various signaling pathways. Phosphorylation of nuclear receptors has been implicated in both transcriptional activation and repression, by influencing nuclear localization, hormone binding and cofactor recruitment. The signaling pathways involve growth factors such as EGF (epidermal growth factor) and IGF (insulin-like growth factor), the neurotransmitter dopamine and PKA activators, to name a few (Aronica and Katzenellenbogen, 1993; Aronica et al., 1994; Ignar-Trowbridge et al., 1992; Ignar-Trowbridge et al., 1993; Power et al., 1991).

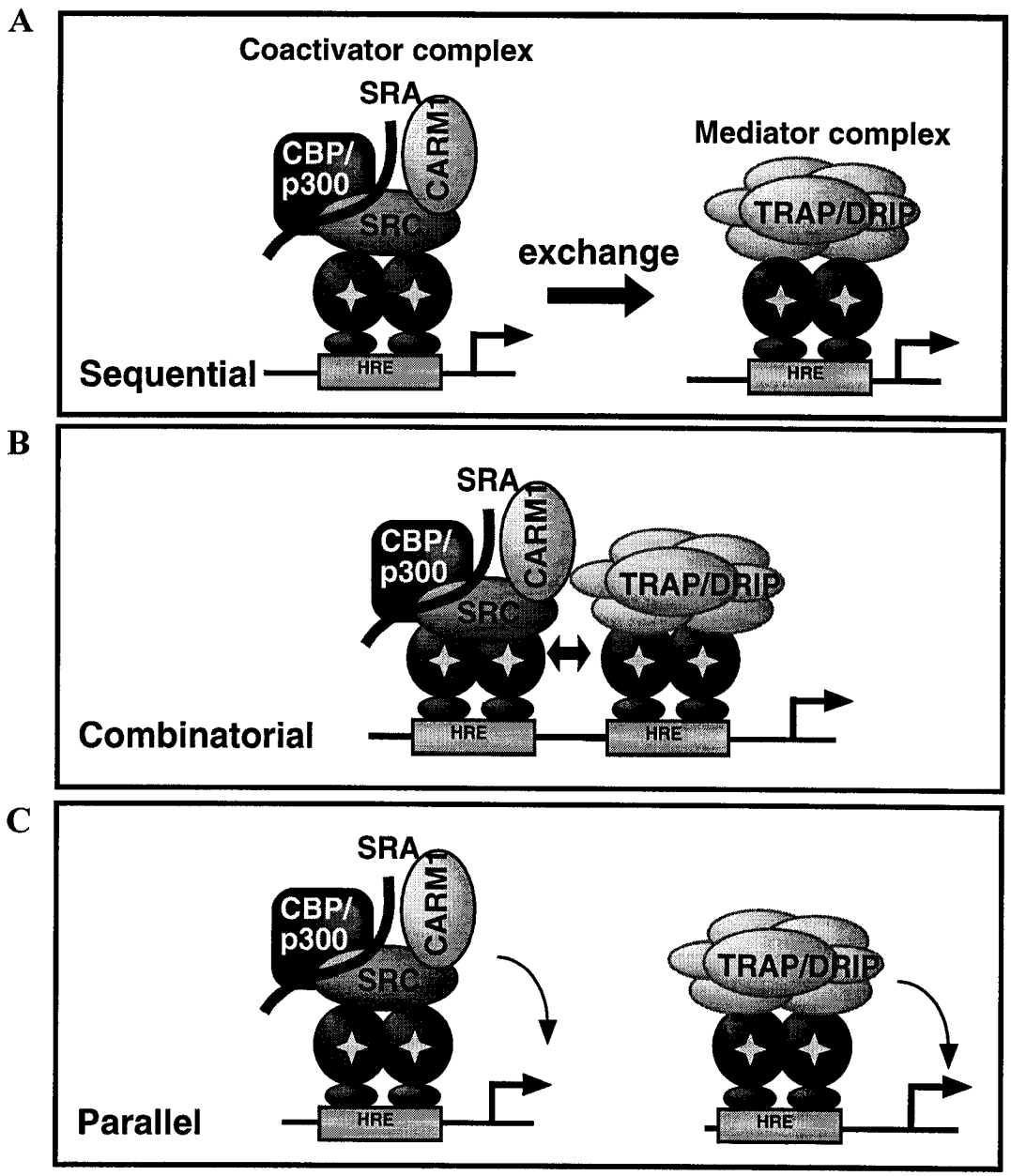


Figure 7. Mechanism of complex recruitment. There are three possible mechanisms: (A) A sequential mechanism involves recruitment of the coactivator complex followed by displacement and recruitment of the mediator complex. (B) In a combinatorial mechanism both coactivator and mediator complexes are recruited simultaneous to the promoter, synergistically activating transcription. (C) A paralllel mechanism invovles independent activation by coactivator and mediator complexes.

The mitogen-activated protein kinase (MAPK) pathway has been shown to be instrumental in the regulation of a number of nuclear receptors (Kyriakis, 2000; Weigel and Zhang, 1998).

The first member of the nuclear receptor superfamily shown to be activated in a ligand-independent manner was the progesterone receptor (PR). Treatment of cells with 8Br-cAMP, EGF, activating the PKA pathway, or okadaic acid, inhibiting phosphatases 1 and 2A, leads to activation of PR-mediated transcription in the absence of hormone (Denner et al., 1990; Zhang et al., 1994). Steroid receptors, with the exception of GR, are activated by a number of signaling pathways in the absence of hormonal stimulation. Hormone-dependent GR activity is potentiated by simultaneous activation of both the PKA and PKC pathways (Nordeen et al., 1994; Rangarajan et al., 1992). The estrogen receptors ERα and ERβ share very little sequence similarity in their NTD domains, yet their transcriptional activities are both potentiated upon phosphorylation of serine and threonine residues of their AF-1 by the MAPK pathway (Kato et al., 1995; Patrone et al., 1996; Tremblay et al., 1999). Phosphorylation of the ERα AF-1 domain induces nuclear translocation in response to estrogen-independent stimulation, facilitating DNA binding (Katzenellenbogen, 1996; Lu et al., 2002). The peroxisome proliferator activated receptor (PPARα) is also modulated by MAPK, which phosphorylates the AF-1 domain and enhances transactivation (Juge-Aubry et al., 1999). Phosphorylation of a serine residue in the AF-1 of the orphan nuclear receptor SF-1 enhances cofactor recruitment, increasing transcriptional activation (Hammer et al., 1999).

In contrast, many nuclear receptors are antagonized by the activation of the MAPK pathway (Shao et al., 1998). For example, PPARγ, upon phosphorylation of its AF-1 domain by SAPK/ERK/MAPK, exhibits decreased ligand binding, which negatively influences transcriptional activation (Camp and Tafuri, 1997; Hu et al., 1996). Phosphorylation of the GR at Ser^{246} by SAPK (stress-activated protein kinase), activated by proinflammatory stimuli, inhibits GR agonist (dexamethasome)-induced transcriptional activation (Rogatsky et al., 1998). ERK-catalyzed phosphorylation of RXRα blocks vitamin D_3 from activating its heterodimerization partner VDR (Solomon et al., 1999).

Nuclear receptor activity is also regulated during the cell cycle. RARs and other nuclear receptors are phosphorylated by cyclin-dependent kinases (CDKs) influencing both their ligand-dependent and ligand-independent transactivation (Rochette-Egly et al., 1997; Rochette-Egly et al., 1991). The AF-1 domains of GR, PR and RAR nuclear receptors have been shown to be regulated by CDK phosphorylation and ER is regulated by $pp90^{rsk1}$ (Joel et al., 1998; Krstic et al., 1997; Rochette-Egly et al., 1997; Rochette-Egly et al., 1992; Zhang et al., 1997).

Coregulators, aside from directly mediating repression or activation, serve as integrators of signals emitted from transcription factor in responses to various stimuli, and can be directly targeted by signal transduction pathways. Activation of the MAPK pathway has been shown to mediate the redistribution of SMRT corepressor within the cell, from a predominantly nuclear to a perinuclear or cytoplasmic localization (Hong and Privalsky, 2000). Moreover, activation of MAPK modulates the dissociation of corepressors from nuclear receptors, demonstrated by the

inhibition of NcoR interaction with antagonist-bound ER due to phosphorylation of the ER NTD (Lavinsky et al., 1998). In contrast to the redistribution of SMRT in the cell in response to MAPK, phosphorylation of SRC family members leads to relocalization from the cytoplasm to the nucleus (Wang et al., 2000). The HAT activity of CBP on the other hand, is regulated by Cdks during the cell cycle (Ait-Si-Ali et al., 1998). Signal transduction pathways add another layer of regulation not only to nuclear receptor function but to coregulator function as well.

9. The Ubiquitin-Proteasome Pathway Regulates Nuclear Receptor Transcription

Nuclear receptors are short-lived ligand-inducible transcription factors whose turnover is mediated by the ubiquitin-proteasome complex. In addition to transcription factors, the turnover of many short-lived proteins such as cell growth modulators, signal transducers, and cell cycle proteins, as well as damaged, misfolded or abnormal proteins occurs through the ubiquitin-proteasome pathway (reviewed in Ciechanover, 1998; Glickman and Ciechanover, 2002; Voges et al., 1999). Proteasomal targets may be cytosolic, nuclear, membrane-anchored, or secretory pathway compartmentalized proteins. Given the wide range of substrates, the ubquitin-proteasome complex plays an important role in many cellular processes such as: regulation of cell cycle, differentiation and development, cellular response to extracellular effectors and stress, modulation of cell surface receptors and ion channels, DNA repair, modulation of the immune and inflammatory responses, control of signal transduction pathways, development and differentiation, biogenesis

of organelles and apoptosis. Moreover, it plays a very important role in regulating transcription and is emerging as a key regulator of eukaryotic mRNA synthesis (Laroia et al., 1999).

The ubiquitin-proteasome complex. Ubiquitin (Ub) is a small 76 amino acid protein found throughout the cell in a free form or complexed with other proteins, and is highly conserved from yeast to man. It is conjugated to proteins via a reversible covalent bond between the carboxy ternimus of Ub and lysine side chains in the target protein. Degradation of a protein by the ubiquitin system involves two distinct and successive steps: ATP-dependent covalent attachment of multiple ubiquitin molecules to the target protein and degradation by the 26S proteasome. The 26S proteasome is a large muticatalytic protease composed of a core 20S catalytic particle and a regulatory 19S particle that recognizes the ubiquitinated substrate (Figure 8). The 26S ATP-dependent proteolytic complex degrades proteins into small peptides of 3-20 residues followed by hydrolysis into single amino acid residues. Proteins are marked for degradation by the covalent attachment of a macromolecular ubiquitin chain. Conjugation of Ub to its target proteins is mediated by the serial action of three types of enzymes: E1 enzyme, or Ub-activating enzyme, modifies Ub so that it is in a reactive state; E2 enzymes, or Ub-conjugating enzymes (Ubc), catalyze the attachment of Ub to the substrate protein; and E3 enzymes, or Ub-ligases, provide specificity by recognizing the substrate, serve as a scaffold between E2 and the substrate, and function in concert with the E2 enzymes. Conjugation of ubiquitin to target proteins is not irreversible, and can be cleaved by deubiquitination enzymes. These enzymes are essential for the maturation of newly synthesized Ub molecules,

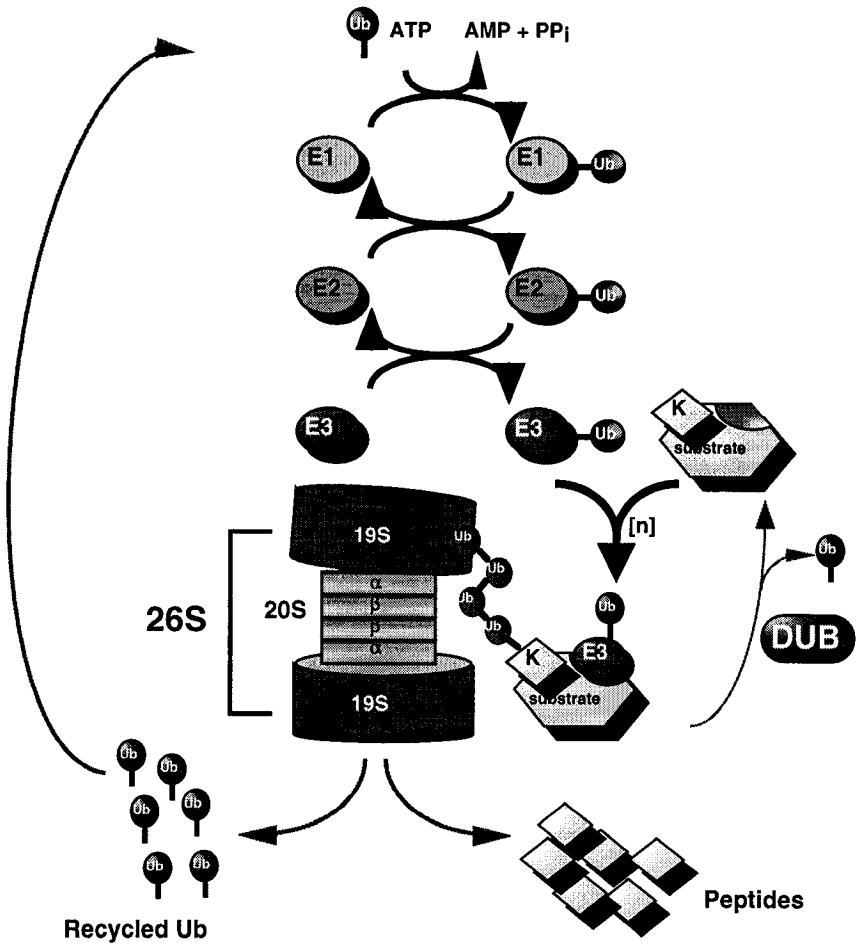


Figure 8. Degradation of target proteins by the ubiquitin-proteasome complex. Ubiquitination is mediated by the serial action of three enzymes requiring ATP hydrolysis: E1, activating enzyme; E2, conjugating enzyme; E3, ubiquitin ligase. Ubiquitin moieties are attached to lysine residues of substrate proteins. The reaction is carried out repeatedly as to create a polyubiquitin chain. The 26S proteosome consists of 19S recognition subunits and 20S catalytic subunits degrading a polyubiquitinated protein into peptides releasing ubiquitin to be recycled. Ubiquitination is reversible and mediated by deubiquitinating enzymes (DUBs).

for the release of ribosomal proteins that are fused with Ub when synthesized, and for the recycling of Ub molecules after the degradation of substrate. Inactivation of the deubiquitinating enzymes (DUBs) inhibits proteasomal degradation due to a depletion of free Ub molecules (Figure 8).

Degradation of substrates by the ubiquitin system apparatus is mediated by specific degradation signals, termed 'degrons', consisting of sequences or structural motifs on the substrate. The N-end rule dictates that the first amino acid at the N-terminus of the protein can be used to predict the protein's half-life and is an E3 recognition determinant triggering proteolysis (Varshavsky, 1997). The PEST sequence has also been shown to function as a signal for degradation. It is a short stretch of about 8 amino acids enriched with proline, glutamic acid, serine, and threonine residues that often contains a phosphorylation site flanked by lysine, arginine or histidine (Rechsteiner and Rogers, 1996; Rogers et al., 1986). PEST sequences are also often coupled with activation domains in transcription factors. The stability of a protein also depends on its oligomerization and phosphorylation states, or association with other proteins such as molecular chaperones that act as recognition elements. Phosphorylation often acts as a degradation signal for substrates whose degradation rate must be tightly coupled to the cellular environmental status or cell cycle stage. Hydrophobic surfaces exposed in non-native proteins or the absence of a protein partner may also signal degradation.

In humans, mutations in the ubiquitin-proteasome system can lead to a number of diseases including: Angelman's syndrome, which involves a mutation in the ubiquitin-ligase E6-AP and results in severe motor and intellectual retardation; and

Liddle's syndrome, in which a mutation in the kidney epithelial sodium channel (EnaC) preventing interaction with the ubiquitin ligase Nedd4, leads to severe hypertension. Upregulation of the proteasomal pathway for bulk degradation of skeletal muscle can occur during normal fasting conditions, in addition to pathological conditions such as cancer cachexia, severe sepsis, metabolic acidosis, or denervation (reviewed in Kornitzer and Ciechanover, 2000).

The role of the proteasome in transcription. RNAP II and a growing number of transcription factors interact with a large number ubiquitin ligases and are targeted for ubiquitinylation followed by destruction. A single transcription factor can be regulated by multiple ubiquitin-ligases ensuring degradation and cessation of transcription. Ub-mediated proteolysis is also involved in processing inactive precursors to active forms of transcription factors. For example, NF-κB precursors p105 and p100 are processed by $SCF^{\beta\text{-}TRCP}$ ub-ligase which triggers their C-terminal proteolytic cleavage into their active subunits p50 and p52, respectively. For other transcription factors, ubiquitinylation and transcriptional activation are closely coupled events, whereby a correlation between the instability of a transcriptional activator and the potency of its activation domain has been established. Molinari *et al* fused activation domains to Gal4 DNA binding domain and demonstrated that the more potently the given fusion activated transcription the more rapidly it was degraded. It was also shown that acidic activation domains such as VP16 are protein destabilization domains (Salghetti et al., 2001; Salghetti et al., 2000). Linkage between the transcriptional activation domain and destruction provides cells with an efficient fail-safe suicide mechanism for attenuating transcription. Ubiquitin ligases

responsible for tagging transcription factors may be an integral component of the RNAP II machinery.

Nuclear receptor stability is influenced by ligand-binding, phosphorylation, interaction with cofactors, and by DNA-binding. ER, PR, RXR, RAR, PPARγ, T_3R, and GR nuclear receptors have been shown to be downregulated in a ligand-dependent manner (Alarid et al., 1999; Boudjelal et al., 2002; Boudjelal et al., 2000; Dace et al., 2000; El Khissiin and Leclercq, 1999; Hauser et al., 2000; Kopf et al., 2000; Lonard et al., 2000; Nawaz et al., 1999a; Nomura et al., 1999; Prufer et al., 2002; Syvala et al., 1996; Tanaka et al., 2001; Wallace and Cidlowski, 2001; Wijayaratne and McDonnell, 2001; Zhu et al., 1999). Transcriptional activation and proteasomal degradation are closely coupled events. Proteasomal inhibition generally interferes with receptor-mediated transcription, with the exception of the GR where accumulation of this receptor leads to a synergistic increase in transcription (Deroo et al., 2002). Other receptors such as VDR, AR, and PXR are protected from degradation in the presence of their cognate ligands (Li et al., 1999b; Masuyama et al., 2002). The conformational change induced by ligand binding allows cofactor docking, many of which are also components of the ubiquitin-proteasome degradation system, including SUG1/TRIP1 (Suppressor for gal1/T3R-interacting protein), Rsp5/PRF1, and E6-AP (Lee et al., 1995; Rubin et al., 1996; Rubin et al., 1997; von Baur et al., 1996). Hect domain E3 ligases, in addition to their obvious role in targeting steroid receptors for degradation, may also serve as coactivators enhancing transcription mediated by GR, PR, and ER nuclear receptors (Imhof and McDonnell, 1996; Nawaz et al., 1999a; Nawaz et al., 1999b).

Nuclear receptors are targeted for ubiquitin-mediated proteolysis in a MAPK-dependent fashion. For example, activation of the stress-activated protein kinase (SAPK) pathway blunts RXR-mediated transcription. A SAPK-specific MAPK kinase, known as MKK4, phosphorylates RXRα and triggers degradation of the receptor which can be reversed by proteosome inhibitor MG132 (Lee et al., 2000a). Degradation of RARγ2 is signaled by both the AF-1 and AF-2 transactivation domains by phosphorylation via MAPK and recruitment of SUG-1, respectively, in response to retinoic acid (Gianni et al., 2002). PR is also ubiquitinylated and degraded in a ligand-dependent and ERK phosphorylated-dependent manner. Interestingly, under different circumstances degradation of PR can occur in a phosphorylation-independent manner. Mature unliganded PR is complexed with heat shock proteins Hsp90 and chaperones p23, upon disruption of this complex prior to ligand binding, unbound immature PR-B is subject to proteolysis independent of phosphorylation by the MAPK pathway (Lange et al., 2000).

Nuclear receptor coregulators are also targets themselves of proteasomal degradation. There is evidence that cell-specific repression by nuclear receptors correlates with NCoR corepressor protein levels. NCoR protein levels are regulated by mSiah2 protein, a homolog of the *Drosophila seven in abstentia* (sina), implicated in regulating the proteasomal degradation of their target proteins (Zhang et al., 1998a). Members of the SRC family and the CBP co-integrator are also degraded by the ub-proteasome pathway (Baumann et al., 2001; Lonard et al., 2000). Proteasome-mediated downregulation of one coactivator complex may be necessary for coactivator complex exchange.

Proteolysis can be envisaged as a break on nuclear receptor mediated transcriptional activation, ensuring the appropriate hormonal activation. Components of the ub-proteasome pathway are recruited to the promoter region either directly, by a transcriptionally active receptor, or indirectly as part of the activation complex, or by the C-terminal domain of RNAP II, leading to degradation of the activation complex and subsequent cessation of transcriptional initiation. This mechanism tightly regulates transcriptional initiation limiting the duration of promoter occupancy by transcription factors and in concert allowing transcriptional elongation to proceed (Figure 9).

10. Orphan Nuclear Receptors and Their Ligands

The term 'orphan' nuclear receptor was initially coined to describe receptors that share sequence identity with the nuclear receptor family but whose ligand is unknown (Giguère, 1999; Kliewer et al., 1999; Willson and Moore, 2002). Orphan nuclear receptors are probably the most ancient type of receptor given that many of them are found in insects, nematodes, and other nonvertebrates (Enmark and Gustafsson, 1996). The greatest challenge in studying orphan receptors lies in determining whether they are ligand responsive, and if so to identify their cognate ligand. An initial understanding of how a given orphan receptor functions is limited to structural and functional studies based on comparative studies of sequence identity, DNA-binding, dimerization, and transactivation potential. Mouse models are very useful for studying the developmental and physiological processes associated with a given orphan. Information gathered from these functional and genetic studies often provide

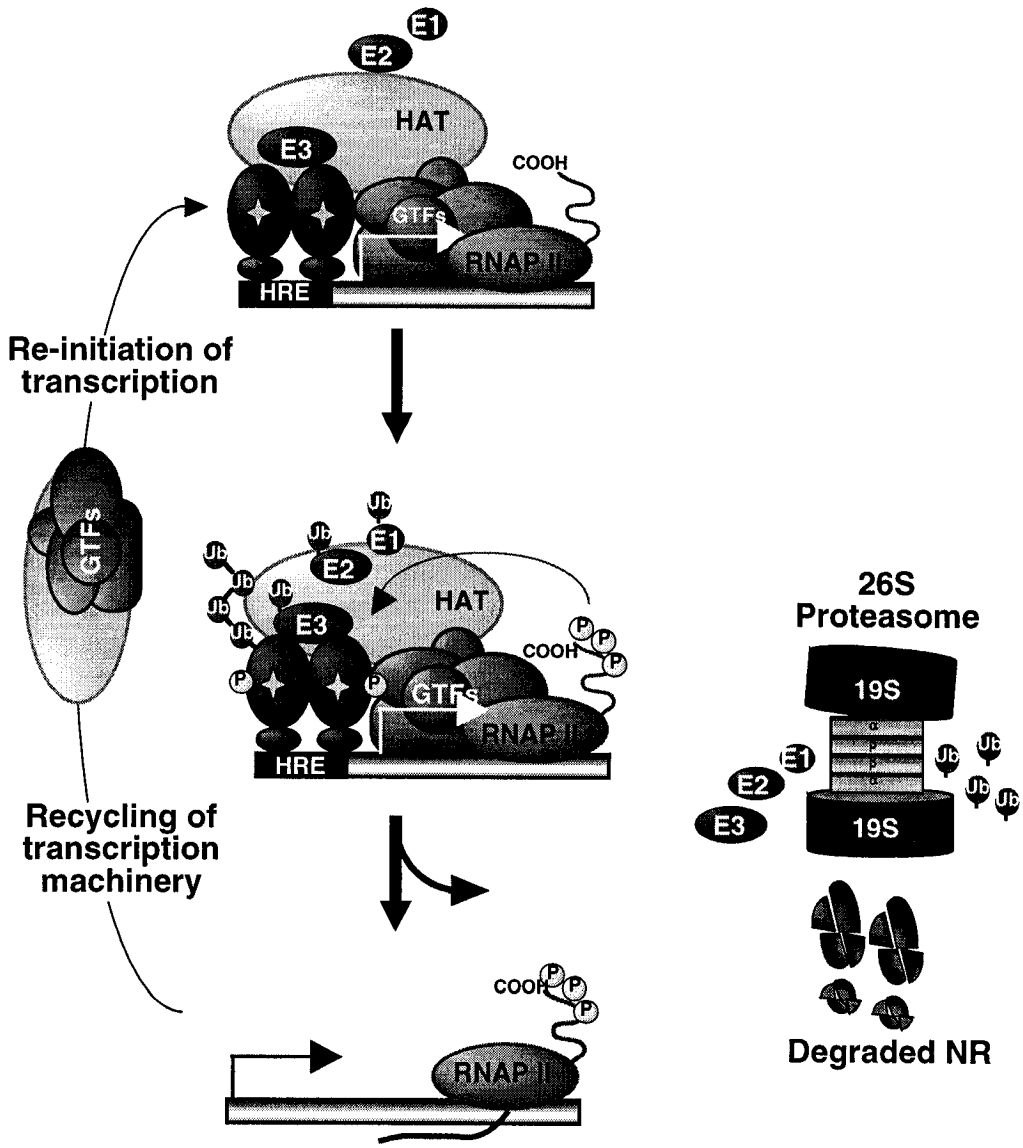


Figure 9. Transcriptional activation is linked to proteasomal degradation. Nuclear receptors recruit ub-ligases (E3) in a ligand- and/or phosphorylation-dependent fashion. Phosphorylation of the C-terminal domain of the RNA polymerase II (RNAP II) also leads to recruitment of E3s. Nuclear receptors and components of the activation complex are degraded by the 26S proteasome, freeing the promoter of the transcriptional initiation complex, allowing for transcriptional elongation to proceed and re-initiation of transcription by recycling of the transcriptional machinery.

important clues as to the physiological or cellular pathways regulated by the receptor in question, and bestow insight as to candidate putative ligands. Knowledge of a receptor's natural ligand is invaluable in understanding its physiological role. Screening a carefully selected set of candidate ligands was fruitful in finding ligands, albeit with low binding affinity, for PPAR, LXR, and FXR. Interestingly, these orphans all share the ability to heterodimerize with RXR suggesting that all RXR partners are regulated by ligands. To date, orphan ligands that have been identified are mainly dietary hormones, intermediates of lipid or cholesterol metabolism or xenobiotic compounds.

There are three members of the PPAR (Peroxisome Proliferator-Activated Receptor) family, PPARα,β,γ {NR1C1, C2, C3}, and each has a very different expression pattern: PPARα is expressed in liver, kidney, heart and muscle; PPARβ expression is ubiquitous; and PPARγ is most abundant in the large intestine, fat cells, and monocytes. They are all induced to varying degrees by eicosanoids, nonsteroidal anti-inflammatory drugs, and leukotriene β4 (LTB4) (Devchand et al., 1996; Lehmann et al., 1997b; Yu et al., 1995). PPARα binds fibrates, a drug widely prescribed for the reduction of high triglyceride levels and the increase in circulating high density lipoproteins, essential for battling coronary artery disease. PPARα is also involved in the regulation of lipoprotein and fatty acid metabolism. PPARγ on the other hand, a key regulator of fat cell differentiation, binds antidiabetic thiazolidinediones (TZDs) and the natural prostaglandin metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) (Forman et al., 1995; Kliewer et al., 1995; Lehmann et al., 1995). TZDs are used for the treatment of type 2 diabetes, to lower glucose levels as

well as circulating levels of fatty acids, suggesting an involvement of PPARγ in both glucose and fatty acid metabolism.

A search for LXR (Liver X Receptor) {NR1H2/H3} ligands led to the discovery that oxidized derivatives of cholesterol serve as activators for this orphan. An increase in dietary cholesterol leads to an increase in hepatic oxysterol levels, which serve as activators of LXR, a cholesterol sensor and regulator of its catabolism (Janowski et al., 1996; Lehmann et al., 1997a). Bile acid metabolism on the other hand is regulated by the FXR (Farnesoid X Receptor) orphan receptor for which bile acids serve as inducers enhancing its interaction with coactivator proteins and regulating key genes in this metabolic pathway (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999).

The cloning of PXR (Pregnane X receptor) {NR1I2} strayed from the conventional biochemical methods utilized to clone most nuclear receptors and was the product of the new genomic era, that is the product of a computer search of expressed sequence tags (ESTs) derived from a liver cDNA library (Kliewer et al., 1998). PXR is predominantly expressed in the intestine and the liver. A search for a putative ligand led to the discovery that synthetic pregnanes (C21 steroids) and glucocorticoid agonists and antagonists, which are all inducers of PXR-mediated transcriptional activity. In response to these candidate ligands, PXR upregulates the expression of the cytochrome p450 CYP3A gene involved in the hydroxylation of steroid hormones, as well as xenobiotic compounds and essential for subsequent detoxification (Lehmann et al., 1998). PXR thus serves as a xenobiotic sensor and is involved in regulating steroid homeostasis. These orphan receptors not only

demonstrate how extensively nuclear receptors are involved in regulating crucial metabolic pathways but more importantly that they can be regulated by intracrine hormones arising from within the same cell.

The CAR (Constitutive Androstane receptor) {NR1I3} receptor, in contrast to other RXR heterodimers discussed so far, is constitutively active in the absence of ligand. It was found that androstanes, testosterone metabolites, act as "reverse agonists", blocking CAR-RXR transcriptional activity and promoting coactivator dissociation (Forman et al., 1998). ERRγ is the first orphan receptor to adopt a transcriptionally active conformation in the absence of ligand, based on the crystal structure of the LBD. ERRγ constitutive transcriptional activity is independent of ligand, although it is antagonized by diethylstilbesterol or tamoxifen, ER agonist and antagonist, respectively (Coward et al., 2001; Tremblay et al., 2001a; Tremblay et al., 2001b).

11. ROR, a Subfamily of Transcriptional Activators

The members of the ROR (Retinoid-related Orphan Receptor) {NR1F} subfamily are potent transcriptional activators. They are monomeric binding transcription factors that regulate the expression of genes involved in a vast array of cellular and physiological process including cerebellar development, development of the retina, regulation of the circadian clock, thymopoeisis, and immune responses.

RORα is a ubiquitous receptor. The first member of the ROR subfamily, RORα, was isolated as part of a screen to identify RAR related nuclear receptors. The DBD of RARα was used as a probe to screen a rat brain cDNA library. This screen yielded

a partial clone encoding the hallmark zinc fingers of nuclear receptor DBD, which was subsequently used to perform high stringency screening of human retina and testis cDNA libraries. Three isoforms of RORα (α1-α3), were obtained containing open reading frames of 1569, 1668 and 1644 nucleotides, and encoded proteins of 523, 556, and 548 amino acids, respectively (Giguère et al., 1994). Another group using reverse transcription (RT)-PCR with degenerate primers stemming from the highly conserved DBD sequence and RNA from human umbilical vein endothelial cells, cloned RORα1 (initially referred to as RZRα) (Becker-André et al., 1993). A fourth isoform, RORα4 was isolated as part of a screen to identify RORα homologues using the DBD of RORα1 to screen a mouse skeletal muscle cDNA library, as well as using an RNA based approach (Matsui et al., 1995). RORα1-4 all originate from a common gene, and are generated by a combination of alternative promoter usage and exon splicing. These isoforms are almost structurally identical, except for their divergent NTDs (Figure 10). Their NTDs dictate their distinct DNA-binding properties. Alternative splicing of the RORα transcription unit leads to the inclusion of an exon encoding the HC2 cytochrome c pseudogene in RORα2. The human *RORA* gene maps to the human chromosome 15q21-q22, in close proximity to the PML gene, which is involved in reciprocal translocation t(15:17) with the *RARA* gene in patients with acute promyelocytic leukemia (Giguère et al., 1995a).

RORα is expressed in a wide number of tissues including heart, brain, lung, kidney, intestine, spleen, muscle, skin, testis, ovary, and peripheral blood leukocytes. The highest level of RORα is found in cerebellum, peripheral blood leukocytes and the skin. In each organ there is an isoform preference suggesting that each RORα

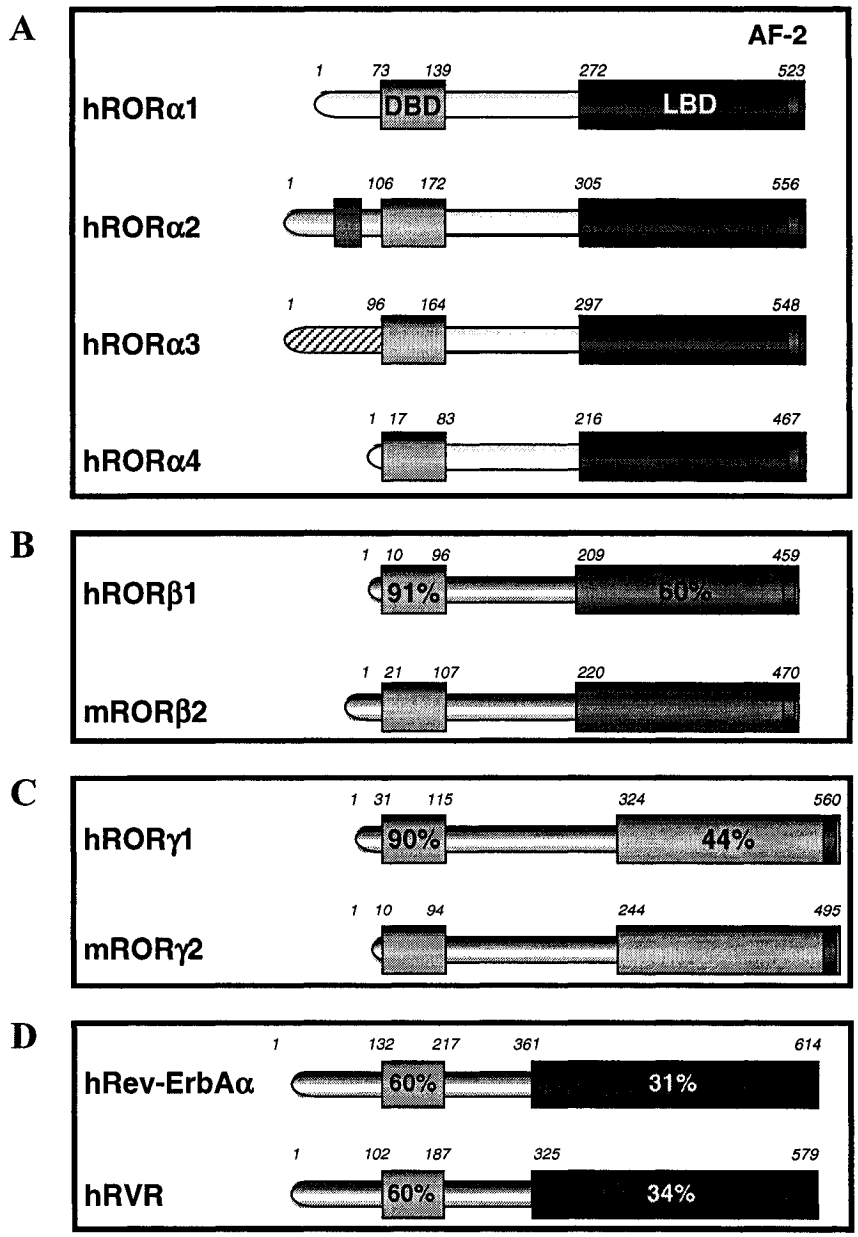


Figure 10. The ROR and Rev-ErbA subfamilies. Schematic representation of (A) RORα, (B) RORβ, (C) RORγ, isotypes and their isoforms RORα1–α4, RORβ1/β2, and RORγ1/γ2 which differ only in their NTDs respectively, as indicated. (D) The Rev-ErbAα and RVR (RevErbAβ) isoforms are encoded by different genes. The % identity shared with RORα1 DBD and LBD is indicated.

isoform may be involved in distinct physiological processes. In the testis, the predominant isoforms are RORα2 and RORα3, which are expressed only after sexual maturation, and are localized to peritubular cells (Steinmayr et al., 1998). RORα1 is predominantly expressed in the thalamus, the suprachiasmatic nuclei of the hypothalamus, and the cerebellum, where expression is restricted to Purkinje cells (Dussault et al., 1998; Hamilton et al., 1996; Steinmayr et al., 1998). RORα4, in addition to expression in cerebellum and leukocytes, is also expressed in skin, particularly the epidermis, hair follicles, and sebaceous glands. Expression in the hair follicle is only observed in the anagen phase of differentiating keratinocytes and is absent in the katagen, telogen and early anagen phases. RORα4 is also expressed in the differentiated, suprabasal layers of the epidermis, suggesting an important role in the regulation of gene expression during epidermal differentiation.

The RORα$^{-/-}$ *staggerer* mouse model. The *staggerer* (*sg/sg*) mutant mouse, generated by a natural recessive mutation that mapped to chromosome 9, was first described 40 years ago (Green and Lane, 1967; Sidman et al., 1962). Homozygote (*sg/sg*) mice are small in size, exhibit body imbalance, and die shortly after weaning. The cerebellar ataxia displayed by the *staggerer* mouse is due to a disruption of the normal development of cerebellar Purkinje cells. Positional cloning using genetic and physical mapping revealed that the *sg/sg* mutation is actually a 6.5kb deletion in the genomic sequence of the RORα gene, including an exon encoding the N-terminal part of the LBD. The deletion also leads to a shift in the open reading frame at amino acid 273 creating a premature stop codon 27 amino acids downstream (Hamilton et al., 1996; Matysiak-Scholze and Nehls, 1997). Targeted disruption of the RORα DBD,

generates mice with a null mutation (ROR$\alpha^{-/-}$), which like the *staggerer* mice, exhibit tremor, abnormal body balance, and are smaller in size than their wild type littermates (Dussault et al., 1998; Matysiak-Scholze and Nehls, 1997; Steinmayr et al., 1998). Their motor coordination, muscle strength and equilibrium capabilities were significantly reduced, based on tests of stumbling frequency, hanging time, and equilibrium, respectively (Steinmayr et al., 1998). The cerebellar cortex of RORα-deficient mice is underdeveloped, the granular layer is nonexistent and is depleted of granule cells, and Purkinje cells are immature and reduced in number. The cerebellum of heterozygote mice appears normal, but there is a significant loss of neurons that occurs during aging. This onset of neuronal loss occurs earlier in males than in females (Doulazmi et al., 1999). Proper development of the cerebellum is also dependent upon the thyroid hormone. Hypothyroidism causes abnormal Purkinje cell neurogenesis as well as decreased granule cell proliferation, a phenotype also seen in *staggerer* mice (Bouvet et al., 1987; Hamilton et al., 1996). However, the phenotype caused by this deficiency can be reversed upon administration of thyroid hormone. Both RORα and T_3R are expressed in Purkinje cells and are likely involved in overlapping signaling pathways crucial for proper cerebellar development.

Despite the strong expression of RORα in the suprabasal layers of the epidermis and hair follicles, ROR$\alpha^{-/-}$ mice do not display any gross morphological defects in either the skin or fur. However, it has been demonstrated that when ROR$\alpha^{-/-}$ mice are shaved, the pelage hair grows back much more slowly and is less dense. Contrary to what would be expected based on the strong expression level of RORα in testis,

RORα$^{-/-}$ mice are fertile and spermatogenesis occurs normally (Steinmayr et al., 1998).

Interestingly, RORα plays an important role in bone metabolism and may be a general regulator of differentiating systems (Meyer et al., 2000). RORα is strongly upregulated during the differentiation of mesenchymal cells into osteoblasts. The bone is a highly metabolically active tissue that is formed by osteoblast cells secreting an organic matrix that is mineralized. The extracellular matrix of the bone is composed of layered type I collagen fibrils, noncollagenous proteins such as the bone sialoprotein (BSP), which modulates mineralization; osteopontin, a protein involved in adhesion; and the bone-specific osteocalcin, which is crucial for proper bone formation. RORα regulates the BSP promoter which encodes a natural RORE element. In contrast, RORα inhibits the activation of the vitamin D-dependent osteocalcin promoter. Osteocalcin functions as a negative regulator of bone formation, which is thus regulated through cross-talk between VDR and RORα orphan nuclear receptor. The absence of a functional RORα receptor in *staggerer* mice leads to a decrease in bone mass demonstrated by the thin long osteopenic bones in comparison to their wild type littermates. This demonstrates that RORα is involved in bone metabolism. The osteopenic phenotype may not be directly induced by the loss of RORα, but rather due to the increased production of IL-1 and IL-6 cytokines in peripheral macrophages of these mice which can stimulate bone resorption leading to osteopenia (Meyer et al., 2000).

RORα is also involved in the proper development of the organs of the immune system. RORα deficient mice exhibit delayed thymus development, a small spleen

and an enlarged lymph node. Helper T cells in these mice are normal, but there is a deficiency in the generation of suppressor cells. Treatment of *staggerer* mice with lipopolysaccharide (LPS) induces higher levels of IL-1α, IL-1β, and TNFα in comparison to treated wild type animals (Kopmels et al., 1992). RORα is expressed in human aortic smooth muscle cells. RORα1 negatively regulates the inflammatory response by interfering with NF-κB signaling. NF-κB drives the expression of IL-6, IL-8 and COX-2 genes, three important cytokines of the inflammatory response. RORα counteracts this by inducing the expression of IκBα, the major inhibitory protein of the NF-κB pathway, via an RORE element in its promoter region (Delerive et al., 2001). RORα$^{-/-}$ mice express lower levels of IκBα in the vascular wall than their wild type counterparts. RORα1 is thus a negative regulator of the vascular inflammatory response, and may serve as a potential target in the treatment of chronic inflammatory diseases, such as atherosclerosis and rheumatoid arthritis.

The RORβ, a brain specific orphan. The second member of the ROR family RORβ (initially named RZRβ) was cloned using an RT-PCR approach on rat brain using degenerate primers based on the sequence of the DBD of known nuclear receptors (Carlberg et al., 1994). RORβ shares a great deal of homology with RORα, but unlike the ubiquitous expression pattern of the primordial ROR, expression of RORβ is restricted to neuronal cells. RORβ is expressed in the brain, several regions of the central nervous system, and in the three principal anatomical components of the mammalian circadian pacemaking system: the pineal gland, the retina and the suprachiasmatic nucleus (Becker-André et al., 1994; Schaeren-Wiemers et al., 1997). RORβ is also involved in processing sensory information, and is expressed

throughout various components of the afferent sensory pathway: receptor organs, the receptive area in the spinal cord, the nuclei in the mesencephalon and brainstem, the thalamic nuclei and cortical target areas. More specifically, within the cerebral cortex, it is exclusively detected in the nonpyramidal neurons of layer IV and V, and is highly expressed in the primary sensory cortices, particularly the primary, visual, auditory, somatosensory, and motor cortex. In the thalamus, expression of RORβ is highest in the sensory relay nuclei, projecting to the primary sensory cortical areas, (Schaeren-Wiemers et al., 1997). It is also found in the receptive area of the sensory pathway in the spinal cord, namely the layers of the dorsal horn that receives sensory input from the periphery. However, RORβ is almost absent in sensory projection neurons such as retinal ganglion cells. It is expressed in retinal progenitor cells in the embryonic rat retina, suggesting a role in the regulation of retinal progenitor proliferation, possibly via the *Chx10* gene that encodes a transcription factor whose absence causes ocular retardation in mice (Burmeister et al., 1996; Chow et al., 1998).

Two isoforms are transcribed from the *RORB* gene using alternative promoters RORβ1 and RORβ2, which differ only in their N-terminal domains (Figure 10B) (André et al., 1998b). RORβ isoforms, as seen with the RORα isoforms, also differ in their respective expression patterns. RORβ1 is highly expressed in the cerebral cortex, hypothalamus and thalamus, whereas RORβ2 is the predominant isoform present in the retina and pineal gland oscillating in a circadian rhythm (Baler et al., 1996; Schaeren-Wiemers et al., 1997). More specifically, RORβ mRNA levels oscillate in a 24hr rhythm in the pineal gland, the primary site of melatonin synthesis, the retina, and the suprachiasmatic nuclei of the hypothalamus under light-dark conditions

(André et al., 1998b; Sumi et al., 2002). Its oscillating expression pattern suggests that this receptor is involved in regulating the circadian timing system. The mammalian circadian rhythm regulates the daily oscillations of the sleep-wake cycle, energy homeostasis, blood pressure, body temperature, renal activity and liver metabolism. A study using daytime and nocturnal animals demonstrated that in the pineal gland the two RORβ isoforms are expressed at different periods of the circadian cycle, with preferential expression of RORβ1 during the day, whereas RORβ2 expression was restricted to the nocturnal animals. The expression of RORβ in the pineal gland is therefore under photoneural regulation, and involves adrenergic and cAMP-dependent mechanisms (Baler et al., 1996).

The RORβ$^{-/-}$ *vacillans* mouse model. The RORβ$^{-/-}$ mouse is a phenocopy of the spontaneous mutant mouse strain *vacillans* described in 1956, now believed to be extinct, that displayed juvenile ataxia with diminished muscular strength and, upon adulthood, exhibited a duck-like gait, which may be due to impaired integration of sensory input information (André et al., 1998a; Sirlin, 1956). RORβ$^{-/-}$ male mice have a developmentally delayed onset of fertility. There is no mechanistic explanation to date for this transient infertility since there are no differences in gene expression between young infertile and old fertile mice. RORβ may play a role in the endocrine network of sexual maturation given that it is expressed in parts of the hypothalamus and in the anterior pituitary gland. Alternatively, loss of RORβ may lead to impaired terminal maturation or inappropriate storage of the sperm cells given its aberrant expression in the epididymis and vas deferens. RORβ$^{-/-}$ mice are blind due to a severely disorganized retina. The malformation of the retina occurs postnatally as a

result of defects in cellular differentiation and a degenerative cell loss. Loss of RORβ in mutant mice leads to an extended circadian period. RORβ may be involved in the transcriptional regulation of effectors of the circadian clock, particularly the regulation of melatonin synthesis, given its expression in the pineal gland and photoreceptors, the two principal melatonin-producing tissues. In addition, the rhythmic expression of RORβ parallels the biosynthesis of melatonin in the retina and the pineal gland (André et al., 1998a).

RORγ, a thymus specific orphan. The use of RT-PCR and degenerate DBD primers, in the search for novel nuclear receptors, led to the cloning of the third member of the ROR subfamily, RORγ (Hirose et al., 1994). The full length RORγ was obtained from a human skeletal cDNA library containing an open reading frame encoding a protein of 560 amino acids, and localized to human chromosome 1. The murine homologue was isolated from a T cell cDNA library while in search of novel receptors that play an immunological role. It is mainly expressed in the thymus and in T cells, hence initially given the name TOR (thymus orphan receptor) (Ortiz et al., 1995). The murine RORγ was cloned from a muscle cDNA library (Medvedev et al., 1996). There are two isoforms of RORγ, RORγ1 and RORγ2. The latter lacks the N-terminal domain but is otherwise homologous to RORγ1 and is therefore a truncated form of RORγ1 also named RORγt, derived from an alternative promoter (Figure 10C) (Villey et al., 1999). RORγ2 was cloned based on a strategy to identify genes that are involved in TCR-mediated apoptosis of thymocytes (He et al., 1998). RORγ2 expression is restricted to the thymus. RORγ1 is detected in thymus, brain, heart, lung, and mammary gland and highly expressed in muscle, kidney, and liver. Neither

RORγ1 nor RORγ2 are expressed in spleen or bone marrow, suggesting that mature T cells or B cells do not express RORγ. RORγ2 (RORγt) is predominantly expressed in immature CD4+CD8+ double-positive (DP) thymocytes, and is absent in mature single-positive (SP) CD4+ or CD8+ cells, or in thymic epithelial cells. Only immature DP cells are sensitive to Fas-induced killing. It has been suggested that in immature thymocytes, RORγ2 inhibits Fas ligand expression and IL-2 cytokine secretion, protecting T cell hybridomas from activation-induced cell death. RORγ2 expression is tightly controlled during thymopoiesis, suggesting that this orphan regulates gene expression at discrete stages of this T cell selection process.

RORγ is highly expressed in brown fat tissue but is not detected in white fat tissue. Both RORγ and RORα are upregulated during adipocyte differentiation (Austin et al., 1998). The TNF-α and TGF-β cytokines, two inhibitors of adipocyte differentiation, suppress RORγ mRNA induction. Moreover, activation of PPARγ, essential for fat cell differentiation, leads to increased RORγ expression. RORγ may play a role in the activation of gene expression required at a late stage of adipocyte differentiation.

The RORγ$^{-/-}$ mouse model. RORγ$^{-/-}$ mice, unlike RORα$^{-/-}$ and RORβ$^{-/-}$ mice, have no obvious physical defect (Kurebayashi et al., 2000; Sun et al., 2000). Despite, their normal appearance and fertility, RORγ deficient mice lack peripheral and mesenteric lymph nodes and Peyer's patches, indicating that RORγ is indispensable for lymphoid organogenesis. Several proteins have been shown to be important in lymph node genesis, among which are the tumor necrosis factor (TNF) family members (lymphotoxin α and β, TRANCE/OPGL/RANKL) and their receptors (TNF receptor

55, lymphotoxin β receptor). Mutants of these genes, except for TRANCE, causes a lack lymph nodes and display disrupted spleenic follicular structure. Id2, a member of the helix-loop-helix family of transcription factors, also plays a role in the development of peripheral lymphoid organs. $Id2^{-/-}$ mice closely resemble $ROR\gamma^{-/-}$ mice, lacking both lymph nodes and Peyer's patches. Id2 and RORγ signaling pathways are important and cross talk between these two transcription factors requires further investigation.

RORγ plays a key role in thymopoiesis. The thymi of $ROR\gamma^{-/-}$ mice are normal in size but have 30-50% less cells than their wild type littermates. Thymocyte survival is decreased and apoptosis is accelerated in $ROR\gamma^{-/-}$, due to a loss of thymic expression of the anti-apoptotic factor Bcl-xL. Expression of an inducible BcL-xL gene in immature thymocytes of $ROR\gamma^{-/-}$ animals restores normal thymocyte development including normal thymocyte survival and cell cycle regulation. Thymocytes from RORγ deficient mice also have decreased amounts of $p27^{kip1}$, a cyclin-dependent kinase inhibitor, and therefore negatively regulates the transition from G1 to S phase. This suggests that RORγ acts upstream of BcL-xL to prolong DP thymocyte survival and promote G1 cell cycle arrest. RORγ plays a role in the negative regulation of apoptosis and promotes cell survival of thymocytes, leading to an overall role in the regulation of homeostasis in the thymus (Sun et al., 2000). $ROR\gamma^{-/-}$ mice have a higher incidence of T cell lymphomas than wild type or heterozygote animals (Ueda et al., 2002). The lack of RORγ results in deregulation of proliferation and apoptosis, disturbing the normal thymocyte maturation, increasing the probability of acquiring mutations and subsequently increasing the susceptibility

of an early onset of T cell lymphoma. RORγ$^{-/-}$ lymphomas have abundant SP CD8^{+} and DP cells, with the lymphoblastic cells frequently metastasizing to the spleen and the liver. Although loss of tumor suppressors p53 or BRCA2, or overexpression of proto-oncogene c-Myc have been shown to promote thymic lymphoma in mice, these proteins are not involved in the development of the lymphoma associated with loss of RORγ. Further investigation into the potential RORγ target genes or other pathways involved in RORγ-mediated regulation of thymocyte development is needed to understand the exact mechanism required for normal thymopoeisis as well as the development of T cell lymphoma.

12. Monomeric DNA Binding

ROR is most closely related to RAR in terms of DBD, sharing a 68% homology. Unlike RAR, ROR does not heterodimerize with RXR. Binding site selection studies revealed that the preferred binding site for RORα, the ROR response element (RORE), consists of a half site encoding the consensus sequence (A/G)GGTCA, preceded by a 6bp AT-rich 5' flanking sequence (Carlberg et al., 1994; Giguère et al., 1994). This RORE may be the primordial response element, from which evolutionary changes led to the generation of tandem repeats. RORs bind ROREs as monomers and do not form homodimers on this response element, nor do they heterodimerize with any other nuclear receptor (Carlberg et al., 1994; Giguère et al., 1994; Greiner et al., 1996; Medvedev et al., 1996). The DNA-binding properties of ROR most closely resemble those of the orphan nuclear receptor Rev-ErbAα, which binds to a response

element indistinguishable from that of ROR, likely regulating overlapping gene networks (discussed below) (Harding and Lazar, 1993).

ROR regulates a great number of different genes each with unique functions implicated in a wide number of cellular processes. A number of potential target genes have been identified based on the identification of an RORE element in their promoter regions including, the cellular retinol binding protein (CRBP) gene, the γF-crystallin gene, the 5-lipoxygenase gene, the laminin B1 gene, the Purkinje cell protein-2 (pcp-2) gene, the bone sialo protein (BSP) gene, the bifunctional enzyme (BFE) gene, the N-myc proto-oncogene, the apolipoprotein AI (apoAI) and CIII (apoCIII) genes, the oxytocin gene, the TEA gene, the $p21^{Waf1}$gene, and the Rev-ErbAα gene (Chu and Zingg, 1999; Coste and Rodriguez, 2002; Delerive et al., 2002a; Dussault and Giguère, 1997; Lau et al., 1999; Matsui, 1996; Matsui, 1997; Schräder et al., 1996; Smith et al., 1991; Steinhilber et al., 1995; Tini et al., 1993; Villey et al., 1999; Vu-Dac et al., 1997). Many of these genes are regulated by a cross-talk between ROR and Rev-ErbA orphan receptors, as well as other nuclear receptors whose HREs either overlap or are independent of the RORE leading to synergistic activation or inhibitory interference (further discussed below).

ROR encodes a bipartite DBD, where two zinc fingers make contacts with the major groove of DNA encoding the RORE half site, and the CTE contacts the minor groove encoding the 5'A/T rich region, demonstrated by methylation and ethylation interference assays (Giguère et al., 1995b). The CTE is critical for optimal monomeric binding and is conserved among ROR isotypes, and Rev-ErbA isotypes, but there is little homology shared with the NGFI-B and SF-1 monomeric orphan

receptors. Although an intact CTE is important for both ROR and NGFI-B, they do not use the same subdomains of their respective CTEs for DNA binding. The NGFI-B A box, consisting of three amino acids, is critical for monomeric binding, while ROR DNA binding on the other hand requires the residues adjacent to the A box (Figure 2B) (Giguère et al., 1995b; Wilson et al., 1991).

RORα isoforms display different binding specificities, owing to their distinct NTDs, which exert both positive and negative influences on DNA binding (André et al., 1998b). RORα1 binds a large subset of ROREs, whereas those recognized by RORα2 are much more limited in sequence variation. Deletion of the NTD of RORα1 reduces its ability to bind RORE. On the other hand, deletion of RORα2 NTD results in increased DNA binding, with a DNA-binding inhibitory region located between amino acids 46-74, which corresponds to the exon encoded on the opposite strand of the cytochrome c-processed pseudogene. The DNA-binding properties of ROR can be conferred to heterologous receptors upon exchange of the NTD, as demonstrated by the ability of T_3Rβ and RAR encoding the NTD of RORα to form monomers with high affinity on a RORE. The NTD also affects the tertiary structure of the receptor when bound to DNA. Circular permutation studies and phasing analysis demonstrated that ROR binding induces a DNA bend towards the major groove at the center of the half-site. Maximal DNA bending is dependent on the hinge region. The NTD influences the DNA bending as well, where deletion of this domain results in a shift of the DNA bend center, suggesting that both the NTD and the hinge regions may be involved in the proper alignment of the zinc finger motifs and the CTE on DNA (McBroom et al., 1995).

13. Are RORs Constitutive Activators or Ligand Inducers?

In transactivation assays, RORs potently activate transcription from a RORE-dependent reporter gene in a number of different cell types (Carlberg et al., 1994; Dussault and Giguère, 1997; Giguère et al., 1994; Greiner et al., 1996; Medvedev et al., 1996; Ortiz et al., 1995). However, RORβ-mediated transcriptional activity is cell-type dependent and is only transcriptionally active in neuronal cells but not in non-neuronal cells (Greiner et al., 1996). One of the most pressing questions when studying an orphan nuclear receptor is whether transactivation is regulated by a ligand. The absence of fetal calf serum from the media does not affect ROR-mediated transcriptional activation in reporter gene assays, suggesting that ROR activity is not dependent on any exogenously added ligand (Ortiz et al., 1995). RORs have been thereby given the title of constitutive activators. Although, the possibility of an intracellular ligand regulating this activity cannot be overlooked. It had been suggested that melatonin is a RORβ ligand, given the high expression of RORβ in the pineal gland, the principal source of this hormone (reviewed in Becker-André et al., 1994; Carlberg and Wiesenberg, 1995; Schräder et al., 1996; Smirnov, 2001). It was shown that RORβ transfected in HeLa cells bound melatonin with a Kd of 5nM, and the half-maximal activation of the reporter gene occurred at an EC_{50} of 3nM, physiological concentrations of melatonin in the bloodstream (Becker-André et al., 1994; Carlberg and Wiesenberg, 1995; Rafii-El-Idrissi et al., 1998). However, melatonin as a putative ROR ligand was quickly disbarred given the irreproducibility of the data (Greiner et al., 1996; Tini et al., 1995). The thiazolidinedione CGP52608

and its derivatives, which are potent antiarthritic agents proven to possess preventive and therapeutic effects in adjuvant-induced arthritis in rats, reported to enhance both RORα and RORβ-mediated transcriptional activity, were also faced with similar turmoil in being accepted as a *bone fide* ROR ligands (Missbach et al., 1996; Wiesenberg et al., 1998). Moreover, based on molecular modeling of RORα using TRβ as a template, docking of either melatonin or CGP53065 into the LBD is not possible without considerable disruption of the LBD (Harris et al., 2002).

Recently, the crystal structure of the monomeric RORβ LBD in an agonist-bound conformation has been resolved (Stehlin et al., 2001). Co-crystallization with a peptide encoding the consensus coactivator LxxLL motif was necessary for the RORβ LBD solubility. RORβ LBD shares 60% and 44% identity with RORβ and RORγ LBD, respectively (Figure 10). Members of the ROR subfamily share the same canonical fold as other nuclear receptor LBDs. RORβ LBD encodes two additional helices the H2', also seen in PPARγ LBD, and H11', which superimposes with loop H11-H12 found in the LBD of many receptors, such as RARγ (Nolte et al., 1998; Stehlin et al., 2001). The predicted sizes of the pockets are also quite different. RORβ LBP is 766Å^3 in size and is the third largest nuclear receptor LBP characterized to date, following PPAR and PXR whose LBP are 1400Å^3 and 1150Å^3, respectively. The human RORα and RORγ LBPs are predicted to be smaller in size, with 568Å^3 and 705Å^3, respectively, suggesting that each isotype may have a different ligand regulating its transcriptional activity.

Mass spectroscopy revealed that the LBP was occupied by a fortuitous ligand, stearic acid. The ligand is necessary to stabilize the LBD, but is apparently devoid of

any transactivation potential. Stearate acid is unlikely to be a natural ligand for RORβ due to the low percentage occupancy of the pocket and its partially disordered conformation. There are 32 residues that line the LBP contributing to ligand binding, 17 of which are conserved in all three isotypes. In each case there are four residues that affect the size of the LBP rendering those of RORα (hRORα/rRORβ: F^{398}/L^{304}, F^{432}/L^{338}, I^{433}/V^{339}, V^{436}/A^{342}) and RORγ (hRORγ/rRORβ: L^{324}/I^{266}, M^{358}/L^{300}, I^{400}/A^{342}, L^{475}/V^{419}) smaller. There are four polar residues that are involved in anchoring the carboxylate group of stearate into the pocket and only one residue the E^{265} varies among the isotypes, where it is replaced by a lysine and a histidine in RORα and RORγ respectively. This suggests that there may be different ligands specific for each ROR isotype where the residue at position 265 will discriminate and allow for specificity. Alternatively, all three ROR isotypes may bind the same molecule in which case the lack of conservation of this amino acid parallels its non-involvement in ligand binding.

14. Coregulators Recruitment by ROR

Molecular modeling of RORα revealed that the hydrophobic cleft essential for coactivator docking is more closely related to T_3R than to RAR, contrary to what would be expected given the higher homology between RORα and RARγ (Harris et al., 2002; Stehlin et al., 2001). SRC coactivators interact with a large number of nuclear receptors, and ROR orphan nuclear receptors are no exception. A yeast two hybrid screen using RORα LBD as bait lead to the identification of SRC-2, TIF-1 coactivators and a component of the mediator complex PBP/TRIP-

2/DRIP230/TRAP220 as RORα-interacting proteins. Other ROR-interacting proteins include: TRIP-1 (also known as SUG-1), a component of the 26S proteosome, and TRIP-11/TRIP230, a retinoblastoma protein- and T_3R-binding protein (Atkins et al., 1999). Interaction with these coactivators occurs in yeast, in bacterial extracts and in mammalian cells both *in vitro* and *in vivo* in the absence of exogenous ligand, in a manner similar to that of ligand-occupied RAR or T_3R nuclear receptors. It is unlikely that yeast harbors a ligand for a higher order eukaryotic nuclear receptor, or that the ligand is found in bacterial extract, suggesting that the RORα interacts with these coactivators in a ligand-independent manner. Moreover, the AF-2 of RORα may be locked in a transcriptionally active conformation enabling ligand-independent coactivator recruitment (Harris et al., 2002). RORα recruits p300 *in vitro* in the absence of ligand reinforcing the view that it is a true constitutive activator (Lau et al., 1999). RORγ interacts with SRC-1 and CBP, which both contain intrinsic histone acetylase activity. RORγ can also recruit RIP-140, which can function both as a coactivator and as a corepressor. In the context of RORγ-mediated transcription, it suppresses this constitutive activity. Similarly, PBP has also been shown to repress ROR-mediated transcription, it has been suggested that they likely compete with endogenous coactivators (reviewed in Jetten et al., 2001).

RORs have earned the title of constitutive activators, but this activity can be attenuated by repression. RORα interacts with both NCoR and SMRT corepressor *in vitro*, although interaction with the latter seems to be inhibited by the AF-2 when the receptor is bound to DNA (Harding et al., 1997). A search for RORβ interacting proteins lead to the isolation and cloning of a 27kD protein termed NIX1 (neuronal

interacting factor X 1), that directly interacts with RORβ, liganded-RAR and T_3R, but does not interact with RXR or steroid receptors. NIX1 binding requires an intact AF-2 domain, resulting in downregulation of nuclear receptor-mediated transcriptional activity in the brain (Greiner et al., 2000). NIX1 does not share any similarities with any known proteins, except for two consensus coactivator LxxLL motifs. Interestingly, the minimal region required for receptor interaction encodes only one of the LxxLL motifs in an inverted orientation (LLQAL aa 87-91). It interacts with DNA-bound nuclear receptors in a ligand-dependent fashion. NIX1 expression is restricted to brain, more specifically to the central nervous system namely in neurons in the dentate gyrus of the hippocampus, the amygdala, thalamic, and hypothalamic regions. A *bone fide* coactivator or corepressor must harbor by definition an autonomous activation or repression domain. NIX1 does not possess either one, and represses nuclear receptor activity likely by interfering with coactivator binding. Two-hybrid assays are a powerful tool in identifying novel protein-protein interactions. This technique has identified the nucleoside diphosphate kinase NM23 as a RORα and RORβ-interacting protein. NM23 has been shown to play a role in organogenesis and differentiation. Interestingly, its expression is inversely correlated with metastasis, however the physiological significance of its interaction with ROR orphan nuclear receptors remains elusive (Paravicini et al., 1996).

15. ROR Regulation by the CamKIV Pathway

Many nuclear receptors are phosphoproteins, whose transcriptional activity and protein stability are both modulated by phosphorylation. RORs have a number of

putative phosphorylation sites and may also be regulated by signal transduction pathways. Both RORα1 and RORγ encode putative protein kinase C (PKC) and protein kinase A (PKA) phosphorylation sites (Giguère et al., 1995b). The AF-2 domain contains a tyrosine residue that may be a target for tyrosine kinases. Mutation of this residue to phenylalanine in RORγ resulted in a loss of interaction with SRC-1 coactivator, thus leading to a loss of transcriptional activation, but interaction with the NCoR corepressor was not affected (Horlein et al., 1995; Oñate et al., 1995).

Intracellular Ca^{+2} plays a very important role in the cell regulating a number of different processes ranging from transcription to cell cycle and apoptosis. This signal is mediated through calmodulin (CaM), a Ca^{+2} receptor found both in the cytoplasm and the nucleus. Ca^{+2}/CaM activates the Ser/Thr CaM-dependent kinases (CaMKs), CaMKI, CaMKII, and CaMKIV which phosphorylate a large number of substrates. Whereas expression of CaMKI and CaMKII is relatively ubiquitous, CaMKIV expression is restricted to the brain, T-lymphocytes, and the spermatogonia and spermatids of the testis. CaMKIV is a nuclear kinase involved in the regulation of transcription, and is activated rapidly upon elevation of intracellular Ca^{+2} levels. It has been shown to regulate CREB (cAMP response element-binding protein), ATF-1 (activating transcription factor-1), and SRF (serum response factor) transcription factors, as well the CBP co-integrator. Moreover, CaMKIV enhances the transcriptional activation of ROR family members RORα1, RORα2, RORγ and to a lesser extent COUP-TF, but does not affect T_3Rα- or ER- mediated transcription. CaMKI but not CaMKII can also stimulate RORα-mediated transcription. Cotransfection of a Ca^{+2}/calmodulin-independent form of CaMKIV and RORα

increases the transcriptional activation from a RORE-driven reporter gene by 20-30 fold. RORα encodes two putative CaMKIV phosphorylation sites in its NTD, surprisingly not required for the kinase induced enhancement of transcription based on mutagenesis analysis, nor is RORα phosphorylated *in vitro* by this Ca^{+2}-activated kinase. On the other hand, the LBD is essential for CaMKIV-induced activation. Interaction between RORα and peptides encoding consensus LxxLL motifs is potentiated in the presence of CaMKIV. These LxxLL motifs are not directly phosphorylated given that they do not encode putative CaMKIV phosphorylation sites, but CaMKIV may be phosphorylating another protein serving as a bridge or scaffold between RORα and the LxxLL peptides, the most likely candidate being p300/CBP. In addition, CaMKIV may phosphorylate a cofactor protein involved in RORα-mediated transcription, or activate a downstream kinase that directly phosphorylates RORα. Alternatively, CaMKIV may regulate the activity of a biosynthetic enzyme involved in the production, modification or even destruction of a naturally occurring RORα ligand. Homologous recombination of the *CaMKIV* locus results in transgenic animals that phenotypically resemble the *Rora* knock-out mice (described below), namely consisting of neurological, immunological and reproductive deficiencies (Ribar et al., 2000). Given these similar phenotypes, the overlapping expression patterns, and the ability of CaMKIV to enhance the constitutive transcriptional activity of RORα likely has physiological significance. However, spermatogesis is greatly affected in $CaMKIV^{-/-}$ mice causing infertility. Thymocytes devoid of CaMKIV activity undergo rapid cell death, reminiscent of thymocytes extracted from $ROR\gamma^{-/-}$ mice (Anderson et al., 1997; Kurebayashi et al.,

2000; Sun et al., 2000). These observations provide a link between CaMKIV and ROR orphan nuclear receptors in the regulation of important biological processes. The mechanism of CaMKIV-mediated activation of RORα constitutive activity remains to be elucidated.

16. Rev-ErbA subfamily, a distant ROR relative

Investigation of the various splice variants of the $T_3R\alpha$ (c-erbA) gene, $T_3R\alpha1$ (r-erbAα-1) and $T_3R\alpha2$ (r-erbAα-2), led to the isolation of a cDNA sequence of a gene encoded on the opposite strand of the $T_3R\alpha$ gene, overlapping with the $T_3R\alpha2$ isoform, named reverse erbA (Rev-ErbAα). Rev-ErbAα is an orphan nuclear receptor that shares a great deal of homology with the DBD, and to a lesser extent with the LBD, of $T_3R\alpha$ and RARα (Lazar et al., 1989; Miyajima et al., 1989). The second member of this subfamily was cloned as part of a screen for RORα-related genes from a mouse brain cDNA library (Forman et al., 1994; Retnakaran et al., 1994). The clone that was isolated from this screen bore a great deal of resemblance to Rev-ErbAα sharing a 97% and 68% identity in the DBD and LBD respectively, and hence was given the name Rev-ErbAα-related receptor (RVR) (also known as Rev-Erbβ/BD73). Both Rev-ErbAα and RVR are widely expressed, with highest level of expression seen in skeletal muscle, brown fat, spleen and the brain.

Despite sharing the same genomic locus with $T_3R\alpha$, and the 57% amino acid identity between their DBDs, Rev-ErbAα binds poorly to $T_3R\alpha$ response elements. DNA binding selection assays revealed that the preferred Rev-ErbAα binding site consists of a 5'A/T rich sequence flanking a T_3R half-site (AGGTCA), bearing a

striking similarity to the RORE response element (Harding and Lazar, 1993). Like ROR, Rev-ErbAα and RVR are monomeric DNA binding proteins unable to heterodimerize with RXR (Dumas et al., 1994). Rev-ErbAα/RVR recognize a second type of response element consisting of a DR2 preceded by a 5'A/T rich sequence (RevDR2), on which they form cooperative homodimers (Harding and Lazar, 1995). The CRBPI gene encodes a natural RevDR2 to which Rev-ErbAα homodimers are recruited, antagonizing retionoic acid-induced RAR transactivation through their common response element (Hall et al., 2002). The crystal structure of the Rev-ErbAα DBD bound to an extended DR2 element was solved (Zhao et al., 1998). There are two major protein-DNA interfaces: residues within the first zinc finger encompassing the P box that contact the major groove encoding the consensus half-site, and residues of the CTE (also referred to as GRIP box) contact nucleotides of the minor groove encoding the 5'A/T rich flanking sequence. The Rev-ErbAα dimer interface involves residues of the second zinc finger and the CTE, unlike steroid homodimers or RXR heterodimers, which don't require residues of the CTE. In addition, the CTE may also provide specificity in terms of the spacing required between two half sites in order to acquire Rev-ErbA binding.

In contrast to ROR, Rev-ErbAα and RVR orphan nuclear receptors constitutively repress the expression of their target genes through both RORE and RevDR2 response elements (Retnakaran et al., 1994). These dominant transcriptional silencers are devoid of the C-terminal AF-2 helix in their LBDs, therefore lacking a functional activation domain (Figure 10D). Their ligand-independent repression is mediated by interaction with the NCoR corepressor and its variants RIP13α and RIP13Δ1 (Burke

et al., 1998). The absence of the AF-2 helix, as demonstrated by homology modeling of Rev-ErbAα/RVR LBDs, leads to exposure of a hydrophobic cleft formed by residues of H3, loop3-4, H4 and H11, essential for NCoR interaction (Renaud et al., 2000). Mutation of these hydrophobic residues abolishes both Rev-ErbAα/RVR interaction with NCoR as well as the ability to repress transcription (Burke et al., 1996; Renaud et al., 2000). The potential ligand binding cavity is filled up by amino acid side chains, leaving a predicted pocket size of 16Å^3 for Rev-ErbAα but not for RVR, suggesting the absence of endogenous ligands. Homology modeling cannot be used to make definite conclusions as to whether a ligand exists for Rev-ErbAα/RVR, although it predicts that these orphans can adopt a low-energy conformation with a pocket that has very little room for a ligand, a seemingly probable conformation given that these receptors are transcriptional silencers.

The Rev-ErbAα knock-out mouse. Despite evidence that Rev-ErbAα is involved in both adipogenesis and myogenesis, genetic ablation of the *ReverbAα* gene results in mice with no obvious phenotype in either fat tissue or skeletal muscle (Chomez et al., 2000). ReverbAα$^{-/-}$ mice are viable and adult animals do not display any striking phenotype other than reduced female fertility. Interestingly, these mutant mice demonstrate cerebellar abnormalities during postnatal development, exemplified by delayed morphological transformations of both Purkinje cells and granule cells. Shortly after birth, Purkinje cells normally begin differentiating and develop a dendritic tree that reaches its maximal length in adulthood. During this critical differentiation period, mice devoid of the *reverb1* gene have Purkinje cells with a much less elaborated dendritic tree in comparison to their wild type littermates.

Similarly, the proliferation of granule cells is maintained past the normal proliferative stage, and their migration from the external granule cell layer to the internal granule cell layer is delayed. In addition, when granule cells do reach the internal granule cell layer in Rev-ErbA$\alpha^{-/-}$ mice, they undergo increased apoptosis.

Interestingly, this neurological phenotype is reminiscent of hypothyroid mice which display a deficiency in the arborization of Purkinje cells, a delay in the proliferation, migration and differentiation of granule cells, as well as increased cell death in the internal granule cell layer. Given the genomic arrangement of Rev-ErbAα and T_3R genes and the phenotypic similarities, one would assume that the Rev-ErbA$\alpha^{-/-}$ mice display a hypothyroid condition due to alterations in the expression of the $T_3R\alpha 2$ transcript. However, mice devoid of Rev-ErbAα have normal thyroid hormone levels as well as $T_3R\alpha 1$ and $T_3R\alpha 2$ expression levels, and the cerebellar abnormalities observed in these mice are due solely to the lack of this transcriptional silencer.

Moreover, the cerebellum of Rev-ErbAα deficient mice morphologically resemble those of RORα knock out mice. The key difference lies in the absence of the ataxic phenotype of Rev-ErbA$\alpha^{-/-}$ mice, which also have a normal number of Purkinje cells that do not undergo cell death. Both orphan receptors are essential for the proper development of these cells but they are likely required at different times. RORα is expressed in Purkinje cells at 14dpc, whereas expression of Rev-ErbAα begins only during the final stages of cerebellar development at P10 (Dussault et al., 1998; Hamilton et al., 1996). It is tempting to speculate that RORα is required to initiate proper Purkinje cell development and that Rev-ErbAα is essential for mediating the

final maturation of these cells. Given that these orphans recognize overlapping response elements, Rev-ErbAα may be required at a later stage to silence the transcriptional activity of RORα-induced genes. The Purkinje cell protein-2 (pcp-2) gene is a likely candidate given it encodes a putative RORE, allowing for cross-talk between RORα and Rev-ErbAα, although its exact function in Purkinje cells remains obscure (Matsui, 1997; Schräder et al., 1996). The expression of this gene is also delayed in hypothyroid rats, suggesting that RORα, Rev-ErbAα and T_3R pathways are involved (Strait et al., 1992). The *reverbl* gene itself is another likely candidate for cross-talk between RORα and Rev-ErbAα in Purkinje cells, where RORα has been shown to upregulate the expression of this gene and Rev-ErbAα autoregulates its own expression by opposing the effects of RORα. The proper balance of RORα and Rev-ErbAα expression levels is crucial for the proper maturation of Purkinje cells, for the proliferation of neuronal cells of the external granule cell layer, as well as the survival of neurons of the internal granule cell layer (Chomez et al., 2000).

Rev-ErbAα is also involved in determining the period length and the phase-shifting properties of the mammalian circadian clock (Preitner et al., 2002). ReverbAα deficient mice exhibit shorter and more diversified period length in comparison to wild type littermates. The circadian clock is regulated by PAS helix loop helix transcription factors BMAL1 and CLOCK that activate the transcription of the cryptochrome (CRY) and period (PER) genes. In a negative feedback loop, CRY and PER attenuate BMAL1/CLOCK-mediated transcription of their own genes. The *Bmal1* promoter contains two putative ROREs, suggesting that it may be regulated by either RORβ or Rev-ErbA orphan nuclear receptors. Rev-ErbAα represses the cyclic

expression of the *Bmal1* gene, and to a lesser extent Clock, both in the suprachaismatic nuclei and in the liver. In turn, BMAL1 and PER oppositely regulate Rev-ErbAα expression. CLOCK and BMAL1 are positive regulators of Rev-ErbAα mediating their effects through three E boxes encoded in the promoter region. In contrast, PER silences the expression of Rev-ErbAα. Rev-ErbAα may be the molecular link in the feedback loop between BMAL1/CLOCK and PER/CRY mediators of the circadian clock (Preitner et al., 2002).

17. Cross-Talk between ROR and Rev-ErbA Orphans

ROR and Rev-ErbA orphan nuclear receptors are linked by the convergence of their overlapping HREs. They provide an on/off switch to a number of genes regulating diverse cellular and metabolic pathways. These orphans not only interfere with each other, but cross-talk has also been observed with other nuclear receptors. For example, the lens-specific γF-crystallin gene is regulated by RAR, T_3R and RORα nuclear receptors through a common HRE recognized by all three receptors. RORα constitutive activity is suppressed by RARα/RXR heterodimers, which also exert a dominant role over T_3R regulation of the γF-crystallin gene (Tini et al., 1995). Similarly, the CRBP and laminin B1 genes are regulated by both ROR and RAR (Matsui, 1996; Smith et al., 1991).

The gene encoding the neuropeptide oxytocin is activated by the RORα1 orphan nuclear receptor through two ROREs encoded in its promoter region (Chu and Zingg, 1999). Expression of this gene is hormonally induced by estrogen, thyroid hormone, and retinoic acid. Moreover, it is negatively regulated by COUP-TFI/II {NR2F1/F2},

Ear-2 (ErbA-related receptor 2) {NR5A1} and SF-1 orphan nuclear receptors. The oxytocin hormone induces specific reproductive behaviors, uterine contractions, milk ejection, natriuresis and vasodilation. The oxytocin gene, expressed in hypothalamic neuroendocrine cells and the pineal gland, is likely regulated by RORβ given their coexpression. Whereas in non-neuronal cells this gene may be regulated by other members of the ROR subfamily (Chu and Zingg, 1999).

A search scanning databases for genes encoding a consensus RORE led to the identification of a putative RORE in the first intron of the N-myc proto-oncogene. Unlike most intronic sequences, the region encoding the RORE is highly conserved between human and mouse, suggesting that this may an important regulatory or enhancer region for this gene. Transcription of the N-myc proto-oncogene is potently activated by RORα1 and down-regulated by RVR. Ablation of the RORE enhancer element increases the oncogenic potential of N-myc, demonstrated by a rat embryo fibroblast transformation assay. In this assay, expression of N-myc was inhibited by RVR, hence behaving as a tumor suppressor. RORα on the other hand potentiated the oncogenicity of N-myc. Deregulation of N-myc, as seen in certain types of neoplasia such as retinoblastoma, neuroblastoma, and small cell lung carcinoma, may involve overexpression of RORα1 or loss of RVR (Dussault and Giguère, 1997).

Thyroid hormone signaling through the T_3R receptor induces myogenesis and activates the muscle-specific bHLH transcription factors, the MyoD gene family (myoD and myogenin), transactivators that direct cell fate, repress cell proliferation and activate differentiation. MyoD induces expression of the cdk inhibitor $p21^{waf1/Cip1}$ promoting cell cycle exit of differentiating cells. RORα is expressed in skeletal

muscle and is constitutively expressed during the differentiation of proliferating myoblast cells to post-mitotic myotubes that have acquired a contractile phenotype. RORα is critical in the control of myogenesis, where cross-talk between RORα and MyoD, by direct interaction of these two proteins, regulates differentiation. RORα also mediates the induction of the cdk inhibitor $p21^{Waf1/Cip1}$ required for myogenesis (Lau et al., 1999). In contrast, Rev-ErbAα and RVR antagonistically regulate muscle differentiation, and inhibit the expression of both MyoD and $p21^{waf1/Cip1}$ genes (Burke et al., 1996; Downes et al., 1995). Rev-ErbAα and RVR are expressed in myoblast cells but their expression is suppressed upon differentiation into post-mitotic multinucleated myotubes. Overexpression of Rev-ErbAα or RVR in myogenic cells leads to inhibition of differentiation, and repression of MyoD and cdk inhibitor $p21^{Waf1/Cip1}$(Burke et al., 1996; Downes et al., 1995). Myogenesis is controlled by the cross-talk between T_3R, ROR and Rev-ErbA.

The development of coronary artery disease is inversely correlated to plasma concentrations of high density lipoprotein (HDL) and apolipoprotein AI (ApoAI), it's a major HDL components. Overexpression of apoAI leads to an increase of HDL, providing protection against atherosclerosis. RORα1 increases the transcriptional activity of the apoAI gene through a functional RORE encoded in its promoter region (Vu-Dac et al., 1997). *Staggerer* mice fed a high fat diet develop severe atherosclerosis, with lesions in the small and large coronary arteries. They also display hypoalphalipoproteinemia, associated with decreased plasma levels of the major HDL protein apolipoprotein (apo) AI and apoAII (Mamontova et al., 1998).

The expression level of the ApoAI is decreased in the small intestine but not the liver of *staggerer* mice.

Apolipoprotein CIII (apoCIII) plays a very important role in plasma triglyceride metabolism. An increase in the synthesis of apoCIII leads to hypertriglyceridemia, positively correlated with artherosclerosis and coronary artery disease. In humans, apoCIII is synthesized in the liver and minimally in the intestine. Identification of factors that decrease the expression of the apoCIII is of considerable interest for the treatment of hypertriglyceridemia. RORα1 activates the expression of the apoCIII gene through two functional ROREs (Besnard et al., 2001). Genetic ablation of RORα in *staggerer* mice leads to decreased apoCIII levels and subsequently decreased plasma triglyceride levels compared to wild type mice (Raspe et al., 2001). Rev-ErbAα antagonizes the RORα-mediated transcriptional activity of the apoCIII gene through a shared RORE located in the proximal promoter (Coste and Rodriguez, 2002). There is another putative RORE in the distal promoter that also serves as an enhancer for apoAI, but Rev-ErbAα binds very weakly. The net transcriptional response of apoCIII is dependent on the relative levels of RORα and Rev-ErbAα. RVR can also repress transcription of this target gene. Rev-ErbAα deficient mice show increased apoCIII mRNA levels. Rev-ErbAα expression is regulated by both treatment with fibrates and glucocorticoids (Gervois et al., 1999; Vu-Dac et al., 1998). Fibrates are widely used hypolipidemic drugs that lower plasma cholesterol and triglycerides. Fibrates increase hepatic expression of Rev-ErbAα through PPARα-mediated activation, which may be the mechanism by which fibrates indirectly cause a suppression of apoCIII. Similarly, treatment of rats with

dexamethasome (Dex) has been shown to result in a decrease of hepatic Rev-ErbAα mRNA levels. This may explain the observed increase of apoCIII levels in mice, as well as the elevation in plasma triglycerides in humans treated with dexamethasome.

The promoter of the Rev-ErbAα gene encodes two putative DNA binding sites including a RevDR2 element through which Rev-ErbAα negatively autoregulates its own transcription (Adelmant et al., 1996). In addition, upregulation of Rev-ErbAα mRNA has been observed upon treatment of hepatocyte cells with fibrates (Vu-Dac et al., 1998). This occurs through the PPARα receptor which heterodimerizes with RXR and activates transcription of the Rev-ErbAα gene via the RevDR2 (Gervois et al., 1999). RORα and Rev-ErbAα share similar tissue distribution patterns, particularly in muscle where they are both induced during myogenesis and are highly expressed in adult skeletal muscle. In myoblastic cells, ectopic expression of RORα leads to an increase in Rev-ErbAα mRNA levels, suggesting that RORα may also regulate expression of the Rev-ErbAα gene (Delerive et al., 2002a). This was further supported by the decrease in Rev-ErbAα gene expression in the skeletal muscle of *staggerer* mice in comparison to wild type mice. In addition to the RevDR2 site, the second putative DNA binding site consists of a 5'A/T rich region preceding a core half site motif forming a functional RORE selectively recruiting RORα1. The Rev-ErbAα gene is a RORα1 specific target gene, whose RORα1-mediated transcriptional activity is potentiated by GRIP1/TIF2 coactivator, also expressed during myogenesis, but not by SRC-1 (Delerive et al., 2002a).

18. Experimental Rational: How is RORα transcriptional activity regulated?

The RORα orphan nuclear receptor subfamily regulates a myriad of genes involved in a vast array of cellular and physiological pathways including cerebellar development, myogenesis, bone metabolism, lipid and lipoprotein metabolism. RORα is a constitutively active receptor that regulates an overlapping gene network with the Rev-ErbA subfamily of repressors. A great deal of controversy has surrounded both the DNA binding mode and the regulation by a putative ligand of RORα. Attenuation of RORα-mediated transcription has only been demonstrated to date to be mediated by passive repression through competition with Rev-ErbAα/RVR receptor. However, the molecular mechanisms regulating RORα transcriptional activation or repression are elusive, and determination of these has been the goal of this thesis study. Although we mainly focus on RORα, given the high homology shared between the three ROR isotypes, structural and functional studies can be extrapolated to RORβ and RORγ. In the first part, we study the DNA binding mode of RORα, and the molecular determinants required for monomeric versus dimeric binding, distinguishing the closely related RORα and Rev-ErbAα orphan nuclear receptors. The second part of this study addresses the molecular mechanisms that govern RORα constitutive activity. Our goal was to determine if a putative ligand regulates RORα transcriptional activity, and examine the role of coregulator proteins. We generated mutations in the ligand binding domain, impairing putative ligand binding and coregulator binding, and addressed their impact on RORα-mediated transcription. We also examined the role of the Hairless corepressor, a T_3R-specific corepressor,

strongly expressed in the cerebellum. The third part examines the role of the ubiquitin-proteasome pathway in both RORα protein stability as well as transcriptional activity. The ubiquitin-proteasome pathway is emerging as a key regulator of transcription and mRNA synthesis, establishing a close link between nuclear receptor degradation and transcription.

19. References

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Chapter II. Transition from Monomeric to Homodimeric DNA-Binding by Nuclear Receptors: Identification of Rev-ErbAα determinants required for RORα homodimer complex formation

Preface

The related orphan nuclear receptors, RORα and Rev-ErbAα share highly homologous DNA binding domains, and recognize overlapping hormone response elements with high affinity. While both receptors avidly bind to a RORα response element (RORE) as monomers, Rev-ErbAα also binds as a homodimer to an extended direct repeat (DR2) element (Dumas et al., 1994; Harding and Lazar, 1993; Retnakaran et al., 1994). In this chapter, we used the related orphan nuclear receptors, RORα and Rev-ErbAα, to study the molecular determinants involved in the transition from monomeric to homodimeric modes of DNA binding by nuclear receptors. We identified the four amino acids within Rev-ErbAα involved in copperative homodimer formation, that are absent in RORα. Substitution of these structural determinants in RORa is sufficient to confer homodimer formation on an extended DR2 element. This chapter concludes that RORα is strictly a monomeric binding protein, lacking the appropriate dimerization determinants required for cooperative homodimer binding (Moraitis and Giguère, 1999).

Abstract

Nuclear hormone receptors belong to a class of transcription factors that recognize specific DNA sequences either as monomers, homodimers or heterodimers with the common partner RXR. *In vitro* mutagenesis studies as well as determination of the crystal structure of several complexes formed by the DNA-binding domain (DBD) of receptors bound to their cognate response elements have begun to explain the molecular basis for protein-DNA and protein-protein interactions essential for high affinity and specific DNA binding by nuclear receptors. In this study, we have used the related orphan nuclear receptors RORα and Rev-ErbAα to study the molecular determinants involved in the transition from monomeric to homodimeric modes of DNA-binding by nuclear receptors. While both receptors bind DNA as monomers to a response element containing a core AGGTCA half-site preceded by a 5'-A/T rich flanking sequence, Rev-ErbAα also binds as a homodimer to an extended DR2 element. Gain-of-function experiments using point mutations and sub-domain swaps between RORα and Rev-ErbAα identify four amino acids within Rev-ErbAα sufficient to confer RORα with the ability to form cooperative homodimer complexes on an extended DR2. This study reveals how the transition from monomer to homodimer DNA-binding by members of the nuclear receptor superfamily could be achieved from relatively few amino acid substitutions.

Introduction

The nuclear receptor superfamily consists of transcription factors whose activity is regulated by small lipophilic molecules that include sterols, steroid hormones, vitamin D, thyroid hormone, retinoids, prostanoids and fatty acids (Mangelsdorf et al., 1995). Superfamily members also embody a large group of related proteins, termed orphan nuclear receptors, for which ligand have not yet been identified (Willy and Mangelsdorf, 1998). Nuclear receptors transduce the effects of their ligands mostly through binding to short DNA sequences, referred to as hormone response elements (HREs). HREs are composed of consensus hexameric sequences arranged in tandem as inverted, everted and direct repeats upon which nuclear receptors can bind as homodimers or heterodimers with the ubiquitous partner RXR (Glass, 1994). In addition, a subset of nuclear receptors bind DNA as monomers to a single consensus half-site preceded by a 5'-A/T-rich flanking sequence (Giguère et al., 1994; Harding and Lazar, 1993; Wilson et al., 1991). Functional analysis of mutant receptors coupled with the determination of the crystal structure of several complexes formed by the DNA-binding domain (DBD) of receptors bound to their cognate response elements have begun to explain the molecular basis for protein-DNA and protein-protein interactions essential for high affinity and specific DNA binding by nuclear receptors. Specific recognition of the core half-site sequence is provided by three amino acid residues at the base of the first zinc finger module (the P box) (Danielsen et al., 1989; Luisi et al., 1991; Mader et al., 1989; Umesono and Evans, 1989) while recognition of the 5-A/T-rich flanking sequence present in monomeric HREs is

mediated by contacts between DNA and amino acid residues located in the carboxy-terminal extension (CTE) of the core DBD (Giguère et al., 1995b; Wilson et al., 1993; Wilson et al., 1992). On the other hand, binding specificity for a given homodimer or heterodimer complex is dictated by DNA-dependent dimerization of the two DBD subunits. Spacing specificity is regulated by motifs contained in determinants located in the first and second zinc finger modules as well as in the CTE and the importance of an individual motif in determining half-site specificity depends on the configuration of the HRE (Kurokawa et al., 1993; Perlmann et al., 1993; Predki et al., 1994; Rastinejad et al., 1995; Umesono et al., 1991; Zechel et al., 1994a; Zechel et al., 1994b; Zhao et al., 1998). For example, steroid receptors homodimerize on inverted repeats and strict half-site spacing by 3 bp is regulated by determinants located at the base of the second zinc finger module of the DBD (the D box) (Luisi et al., 1991; Schwabe et al., 1993; Umesono and Evans, 1989). On the other hand, nuclear receptors that heterodimerize with RXR bind with highest affinity to direct repeats (DR) separated by a characteristic number of nucleotides, and spacer discrimination is provided by the CTE of the RXR's partner as well as by distinct usage of dimerization determinants in the first and second zinc finger modules of RXR (Rastinejad et al., 1995).

RORα is an orphan nuclear receptor that was initially cloned based on its similarity to the retinoic acid receptor (RAR) (Giguère et al., 1994). RORα is a monomeric DNA-binding receptor that constitutively activates genes harboring RORα response elements (ROREs). Mouse genetic studies have shown RORα to be encoded by the *staggerer* locus and essential for cerebellar development (Dussault et

al., 1998; Giguère et al., 1995a; Hamilton et al., 1996; Matysiak-Scholze and Nehls, 1997; Steinmayr et al., 1998). The RORα gene generates at least four distinct isoforms that share common DBDs and LBDs but have distinct amino terminal domains (NTDs) (Carlberg et al., 1994; Giguère et al., 1994). Detailed *in vitro* mutagenesis studies has determined that the CTE is required for high affinity DNA-binding and that the distinct NTDs influence how the CTE recognizes the extended 5' A/T-rich flanking sequence present in ROREs (Giguère et al., 1995b), leading to the proposal that the NTD of RORα provides intramolecular interactions necessary to stabilize receptor-DNA interactions (McBroom et al., 1995).

Rev-ErbAα, an orphan member of the superfamily of nuclear receptors, is encoded on the opposite strand of the c-ErbA (T_3Rα) gene (Lazar et al., 1989; Miyajima et al., 1989). DNA-binding studies have independently shown that RORα, Rev-ErbAα and its close relative RVR/BD73 (also known as Rev-ErbAβ) recognize the same monomeric binding site consisting of a half-site AGGTCA motif preceded by a 5'-A/T rich sequence (Dumas et al., 1994; Harding and Lazar, 1993; Retnakaran et al., 1994). However, Rev-ErbAα lacks a typical activation function (AF2) within the ligand-binding domain (LBD), and competition for common binding sites results in down regulation of RORα-induced gene expression (Forman et al., 1994; Retnakaran et al., 1994). The physiological importance of the monomeric binding site has been demonstrated through the characterization of a functional RORE within the N-myc protooncogene transcription unit (Dussault and Giguère, 1997). RORα and RVR have opposite transcriptional effects on the N-myc gene and mutation of the RORE increases the oncogenic potential of the N-myc gene in a rat embryonic

fibroblast transformation assay, suggesting that deregulation of the activity of members of the ROR and Rev-ErbA family could contribute to the initiation and progression of certain types of neoplasia (Dussault and Giguère, 1997). However, Rev-ErbAα has also been shown to bind DNA as a homodimer to an extended DR2 containing the 5' A/T-rich flanking sequence present in ROREs (Harding and Lazar, 1995). The biological importance of the dimeric interaction has been reinforced by the study of Zamir et al. which provides evidence that the Rev-ErbAα dimer, but not the monomeric form, can recruit corepressors and act as an active repressor (Zamir et al., 1997). Recently, the crystal structure of the Rev-ErbAα DBD bound to an extended DR2 was solved (Zhao et al., 1998). The crystal structure demonstrated that the CTE plays an important role in making direct contacts with the 5' A/T-rich flanking sequence of an extended DR2 and confirmed that contacts between the CTE and the core DBD are necessary to stabilize receptor dimers.

Taken together, our current knowledge of RORα and Rev-ErbAα DNA-binding activities demonstrates that these related orphan nuclear receptors can be used as an experimental model to investigate the molecular basis involved in the transition from monomeric to homodimeric modes of DNA-binding by nuclear receptors. In this study, we have used *in vitro* mutagenesis to produce chimeric receptors to dissect the molecular determinants of monomeric and homodimeric DNA binding within the DBD. We demonstrate that by changing a minimum of four amino acid residues, we were able to confer to the RORα DBD the property to homodimerize on an extended DR2 element. These results identify structural determinants necessary for transition from monomer to homodimer DNA-binding by members of the nuclear receptor

superfamily and reveals that this transition can be achieved from relatively few amino acid substitutions.

Material and Methods

Plasmids. DBD peptides were generated by using pairs of oligonucleotide primers, one containing the antisense strand encoding the end of the CTE with a 5' tail containing a stop codon and a *Bam*H1 site, and the other containing the sense sequence beginning 10 amino acids N-terminal to the first cysteine of the core DBD and an *Asp*718 site, for PCR using pCMXhRORα1 (Giguère et al., 1994) and pCMXhRev-ErbAα (Lazar et al., 1989) as templates. The amplified fragments were digested with *Asp*718 and *Bam*H1 and then reintroduced into the *Asp*718 and *Bam*H1 sites of pCMX. The DBD peptides generated are 102 amino acids (ROR) and 103 amino acids (Rev) long. RORα and Rev-ErbAα DBD mutants used in this study were generated using site-directed mutagenesis as described by the Quick Change Site-Directed mutagenesis kit protocol (Stratagene). The nucleotide sequences of all constructs described above were confirmed by sequencing.

Electrophoretic Mobility Shift Assay (EMSA). Coupled *in vitro* transcription and translation with T7 RNA polymerase and TNT rabbit reticulocyte lysate (Promega) was used to synthesize full length RORα and Rev-ErbAα and the truncated DBD peptides from pCMX-based plasmids. Between 1 and 10 μl programmed rabbit reticulocyte lysate was used in DNA-binding reactions as previously described (Giguère et al., 1995b). Samples were loaded onto a 5% for full length receptors or 8% for DBD peptides nondenaturing polyacrylamide gel for full length receptors and DBD peptides respectively, and electrophoresed at 150 V at room temperature. Quantification of dimer and monomer complexes was done using a Bio-Image

Analyzer Bas1000 (Fuji). The following oligonucleotides and their complements were used as probes: RORE, $^{5'}$-TCGACTCGTATAACT**AGGTCA**AGCGTG-$^{3'}$, DR2, $^{5'}$-TCGACTCGTCTAATT-**AGGTCA**GT**AGGTCA**GCGCTG-$^{3'}$; both probes are derived from consensus sequences obtained from binding site selection experiments (Giguère et al., 1994).

Results

Experimental model. Figure 1 schematically represents the structure of the RORα DBD peptide used in this study and its similarity to the Rev-ErbAα DBD. The DBD is subdivided in three domains referred to as zinc finger module 1 and 2 and the CTE. The minimal RORα and Rev-ErbAα DBD constructs used in this study (referred to as ROR and Rev) include the core DBD encoding the two zinc finger modules flanked by 10 amino acids at the N-terminal end and the entire CTE as previously defined (Giguère et al., 1995b). The illustration also depicts determinants previously shown by mutagenesis and crystallographic studies to be required for the formation of homodimeric Rev-ErbAα or heterodimeric RXR/T_3R complexes (Rastinejad et al., 1995; Zhao et al., 1998). Specific amino acid residues that mediate subunit dimerization in these complexes are identified. Residues present in the dimerization determinants and distinct in RORα and Rev-ErbAα constituted targets for our mutagenesis study.

To validate our experimental model, we first tested the binding of full-length RORα1 and Rev-ErbAα synthesized *in vitro* to oligonucleotides encoding the RORE and an extended DR2 element. As expected, both RORα1 and Rev-ErbAα bind the RORE as monomers (Fig. 2A lanes 2 and 3 respectively). In contrast, RORα1 still binds as a monomer on an extended DR2 whereas Rev-ErbAα forms homodimers on this element (Fig. 2A lanes 5 and 6 respectively). Nuclear receptors, which bind DNA as dimers possess dimerization interfaces in both the LBD and DBD. However, the

LBD dimerization interface plays no role in binding site selectivity. Likewise, the dimerization interface in the LBD of Rev-ErbAα is not essential for DR2 recognition and the minimal region required for cooperative homodimer formation on this element is the DBD (Harding and Lazar, 1995). As the DBD appears to play a dominant role in determining Rev-ErbAα DNA binding specificity, we choose to study the properties of the isolated DBDs. As expected, both ROR and Rev DBDs form monomers on a RORE (Fig. 2B, lanes 2 and 3, respectively). In contrast, the ROR DBD binds as a monomer and the Rev DBD preferentially forms homodimer complexes on an extended DR2 element (Fig. 2B lanes 5 and 6 respectively). In order to be able to monitor the monomer to homodimer transition by ROR DBD mutants in future experiments, we determine the fraction (%) of total bound probe that was contained in the monomer and dimer complexes for a range of protein concentrations using a Bio-Imaging Analyzer (Fuji Bas 1000 MacBAS). As ROR DBD concentration increases, a slower migrating homodimeric complex appears (data not shown). As shown in Fig. 2C, homodimer binding of the ROR DBD to the DR2 is non-cooperative, suggesting that the DR2 half sites are progressively filled by protein monomers. Similar results were obtained when DNA binding activity of the ROR DBD was tested on DR1, DR3, DR4 and DR5 elements containing RORE-like 5' A/T-rich sequences (data not shown). For the Rev DBD, the increase in dimer complex formation was more rapid than could be accounted for by additivity alone, demonstrating that the Rev DBD possesses determinants necessary to achieve cooperative DNA binding (Fig. 2C). Therefore, starting with the premise that a homotypic phenotype would be achieved if both of the required dimerization

interfaces are present in the same molecule, we decided to progressively introduce amino acid residues present in the Rev DBD into the ROR DBD and monitor the ability of ROR DBD to homodimerize on an extended DR2.

Three amino acid residues in zinc finger module 1 participate in the monomer to homodimer transition by ROR mutants. We first studied the first zinc finger module and the base of the second zinc finger module (previously referred to as the D-box, (Umesono et al., 1991)) as these determinants were shown to play important roles in dimer formation and HRE recognition in both homodimeric and heterodimeric DNA-receptor complexes. We engineered complete sub-domain swaps between ROR and Rev DBDs by introducing five and four amino acid changes in the first and second zinc finger modules, respectively (Fig. 3A). As expected, the three chimeric ROR/Rev DBD peptides RORm1, RORm2 and RORm3 retain their ability to bind as monomer to the RORE, although a reduction in total binding is observed when the first zinc module is swaped alone (RORm1) or in combination (RORm3) with the D-box of Rev DBD (Fig. 3B and C). On an extended DR2 element, introducing the first zinc finger module of the Rev DBD in the ROR DBD also reduces binding efficacy: interestingly, the chimeric RORm1 peptide efficiently binds DNA as a homodimer (Fig. 3B, lane 10). In contrast, a D-box swap (RORm2) has no significant effect on either DNA-binding affinity or homodimer formation (Fig. 3B, lanes 11). A switch in both zinc finger modules represented by RORm3 does not increase homodimer binding beyond that observed with RORm1 (Fig. 3B, compare lane 10 to 12). Results presented in Fig. 3C demonstrate that a mutated zinc finger

module 1 provides the ROR DBD with a dimerization interface and demonstrate that the D-box does not play an important role in Rev DBD homodimer formation.

A series of mutations in the ROR DBD was generated to identify specific amino acid residues participating in the dimerization determinants within the first zinc finger module (RORm4 to RORm8, Fig. 4A). Ile^{83} is of particular interest as all dimeric receptors surveyed possess either a Phe or a Tyr residue at this position. In particular, these residues were shown to be directly involved in the formation of the heterodimeric T_3R/RXR complex (Rastinejad et al., 1995). On the other hand, the four other divergent amino acid residues between ROR and Rev DBDs had not been shown to be directly involved in making protein-protein contact in any structure solved so far. Surprisingly, introducing either Lys^{79Val} and Ser^{80Ala} mutation simultaneously (RORm4) or Ile^{83Phe} alone (RORm5) both considerably increase dimer formation by the ROR DBD (Fig. 4B and C). Combining both changes in a single mutant (RORm6) further increases the ability of the ROR DBD to bind as a homodimer (Fig. 4B and C). Although RORm6 forms homodimer complexes less efficiently than the Rev DBD, suggesting that additional determinants are needed for protein-protein interactions. Changing the last two amino acid residues of that module (Ile^{88His} and Thr^{89Ala}) in mutant RORm7 has no significant effect on homodimer formation but lowers binding affinity for the extended DR2 as judged by the intensity of the complex relative to the ROR DBD and other mutants (Fig. 4B and C). Combination of the Lys^{79Val}, Ser^{80Ala}, Ile^{88His} and Thr^{89Ala} mutations in RORm8 demonstrates that while the chimeric DBD peptide has a lower binding affinity, it retains the ability to bind as a homodimer.

A single amino acid residue in zinc finger module 2 participates in monomer to homodimer transition by ROR mutants. Determination of the crystal structures of T_3R-RXR DBD heterodimer and Rev-ErbAα DBD homodimer complexes revealed that, of the four amino acid residues involved in protein-protein interactions, only Thr^{120} is divergent between the ROR and Rev DBDs (Rastinejad et al., 1995; Zhao et al., 1998). We therefore decided to target this amino acid for site-directed mutagenesis of the ROR DBD (Fig. 5A). The Thr^{120Ile} mutation (RORm9) increases considerably the amount of dimer complexes formed (Fig. 5B). This mutation was then combined with the three amino acids of the first zinc finger module previously shown to be important for the monomer to homodimer transition. The resulting construct (RORm10) strongly homodimerizes on an extended DR2 with a dimer ratio equivalent to that of the Rev DBD. Therefore, a minimum of four amino acid changes, three in the first zinc finger module (Lys^{79Val}, Ser^{80Ala}, Ile^{88Phe}) and one in the second zinc finger module (Thr^{120Ile}) are required to provide the ROR DBD with the ability to homodimerize on an extended DR2.

Providing full length RORα with a dimerization interface in the DBD is sufficient for cooperative homodimerization. We tested whether the introduction of a dimerization interface in the RORα1 DBD would be sufficient to allow the full length receptor to form homodimers on an extended DR2 element. RORα1 constructs encoding the Rev-ErbAα dimerization determinants of the first and second zinc finger, RORα1m6 and RORα1m9 respectively, were constructed and assayed by EMSA (Fig. 6). At highest protein concentrations, both RORα1m6 and RORα1m9 mutants form two times more homodimer complexes than wild type RORα1 on an

extended DR2 element. The RORα1m10 mutant encoding both the dimerization determinants of the first and second zinc finger modules forms homodimeric complexes slightly less efficiently that Rev-ErbAα.

Discussion

On the basis of their DNA-binding properties, nuclear receptors can be classified into two major groups: monomers, exemplified by orphan nuclear receptors RORα, Rev-ErbAα, SF-1 and NGFI-B, and dimers which include homodimers and heterodimers (Mangelsdorf et al., 1995). Some receptors belong to more than one group. Rev-ErbAα binds DNA both as a monomer and as a homodimer (Harding and Lazar, 1995), NGFI-B as both a monomer and a heterodimer (Forman et al., 1995; Perlmann and Jansson, 1995), while T_3R can bind DNA as a monomer, homodimer and heterodimer with RXR (for references, see Glass, 1994). Homodimeric orphan nuclear receptors such as Rev-ErbAα and HNF4 bind to direct repeat HREs (Harding and Lazar, 1995; Jiang et al., 1995) whereas steroid hormone receptors form homodimers on inverted HREs (Beato et al., 1995). Heterodimeric complexes always involve RXR, and interestingly, RXR's partner is usually associated with a known ligand (Mangelsdorf and Evans, 1995). The flexibility observed in the DNA binding properties of nuclear receptors suggests that, as previously observed for the determinants required for discrimination of HRE sequences (Mader et al., 1989; Umesono and Evans, 1989; Umesono et al., 1991), few changes would be required for a receptor to acquire novel DNA binding characteristics and thus provide a simple mechanism for receptor evolution. The results of this study clearly demonstrate that this may be the case since by changing only four amino acids, the DNA binding mode of the orphan nuclear receptor RORα1 can be converted from monomer to homodimer.

Transition from monomeric to homodimeric DNA-binding by nuclear receptors is facilitated by the dual role played by the CTE in DNA-binding. As described in the introduction, the CTE contains essential determinants for recognition of the 5'-A/T rich flanking sequence of monomeric HRE and in addition participates in the formation of the dimer interface of homodimeric and heterodimeric receptor complexes (Giguère et al., 1995b; Rastinejad et al., 1995; Wilson et al., 1992; Zhao et al., 1998). Thus, one can hypothesize that while keeping intact the highly conserved CTE required for monomeric DNA-binding, progressive evolutionary changes in the zinc finger modules of the DBD could allow nuclear receptors to acquire the ability to bind DNA as homodimers. In fact, significant homodimer binding can be observed with single amino acid changes without significant loss of monomeric DNA-binding (data not shown), indicating that the transition from monomer to homodimer binding could be done progressively without engendering a non-functional receptor. This process, which expands the repertoire of target genes regulated by nuclear receptors, parallels the previously observed non-disruptive changes in the P-box that allow for progressive acquisition of new binding specificity by nuclear receptors (Umesono and Evans, 1989). Alterations in the CTE and zinc finger modules could lead to recognition of novel HREs with distinct half-site spacing. Taken together, these studies illustrate how few changes in common determinants could lead to a wide variety of DNA-binding mechanisms utilized by members of the nuclear receptor superfamily.

While this paper was in preparation, the crystal structure of the Rev-ErbAα DBD was published (Zhao et al., 1998). This study shows that, in contrast to RXR

heterodimer complexes bound to direct repeat HREs, homodimer formation of Rev-ErbAα DBD subunits to an extended DR2 element involves direct contacts between residues in the second zinc finger module of the first subunit with the CTE of the second subunit, but does not involve residues within the first zinc finger module. While our study supports the importance of amino acid residues within the second zinc finger module for homodimerization, it also demonstrates that amino acids within the first zinc finger module are equally important for dimer formation by chimeric ROR DBD peptides. It is possible that the amino acids encoded in the first zinc finger module are not directly involved in protein-protein contacts of the dimerization interface but rather are involved in intramolecular interactions necessary for the proper positioning of other residues involved in forming the dimer interface. It is interesting to note that while position 88 in the first zinc finger module of RORα1 is occupied by an Ile or Val residue within the ROR family, including the *Drosophila* orphan receptor DHR3 shown to bind DNA as a monomer (Horner et al., 1995), the corresponding position in nuclear receptors belonging to group 1 of the nuclear receptor superfamily (Laudet, 1997) is occupied by residues containing aromatic rings (Fig. 7). Members of this subgroup that have a residue with a hydrocarbon sidechain instead of an aromatic ring at this position could be predicted to bind DNA exclusively as monomers. So far, the only nuclear receptor outside of the ROR family to possess this characteristic is *O. volvulus* NHR-1 (Yates et al., 1995), but its DNA binding characteristics have not been investigated. If this observation is supported by future studies, the prediction will be that very few

members of the nuclear receptor superfamily would bind DNA exclusively as monomers.

Dimerization is usually required for DNA-binding by nuclear receptors as it orients and stabilizes adjacent DBDs which are unable to interact in absence of DNA (Glass, 1994). Since RORα lacks key DBD dimerization determinants but nonetheless binds to DNA with high affinity, it must do so in a way that is different from other nuclear receptors. Our previous biochemical and mutagenesis analyses showed that a RORα monomer binds a RORE in a bipartite manner, placing the first zinc finger module into the major groove at the 3' AGGTCA element, and the CTE interacting with the adjacent minor groove at the 5' A/T-rich extension of the RORE (Giguère et al., 1995b). More importantly, these experiments have also demonstrated that intramolecular interactions stabilize the RORα-DNA monomer complex, as the NTD and the nonconserved hinge region cooperate to properly align the zinc finger modules and the CTE with respect to each other (McBroom et al., 1995). While the molecular basis for high affinity monomeric DNA-binding (and for the transition to dimeric DNA-binding) begin to be unraveled, application of direct structural approaches will be required to understand fully the complex intramolecular interactions necessary for RORα and other monomeric receptors to stably and precisely make contacts with their cognate site.

Acknowlegments

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Figure 1

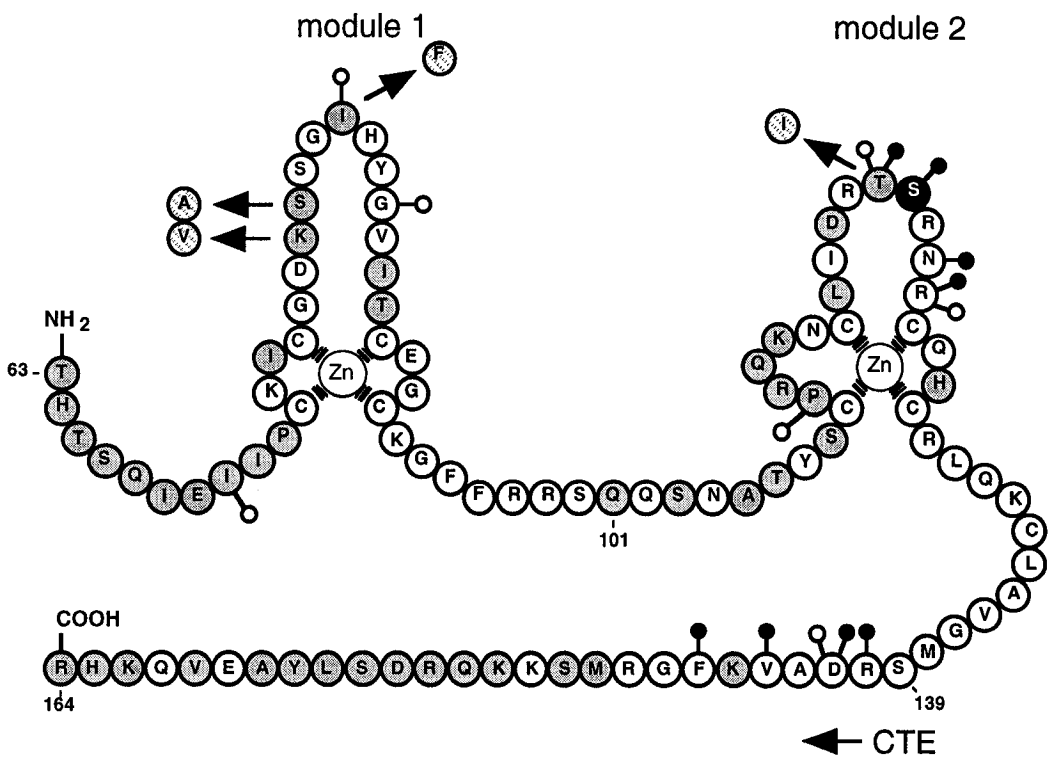

Figure 1. Schematic representation of the RORα DBD and known nuclear receptor dimerization determinants. The DBD of human RORα is divided in three functional subdomains referred to as zinc finger module 1 and 2 and the carboxy-terminal extension (CTE). Numbering is according to the full-length RORα1 (Giguère et al., 1994). Residues that are identical in the RORα1 and Rev-ErbAα sequences are shown in white circles, nonconserved residues are represented in grey circles. The Ser residue represented by a black circle is not conserved within the ROR family. Closed and open symbols linked to amino acid residues represent residues that have been shown to mediate dimerization between partners in the Rev-ErbAα homodimer (Zhao et al., 1998) and RXR-T_3R heterodimer (Rastinejad et al., 1995) complexes, respectively. Arrows point to four amino acid substitutions (stippled circles) that together confer the ROR DBD peptide with the ability to bind as a dimer to an extended DR2 element.

Figure 2

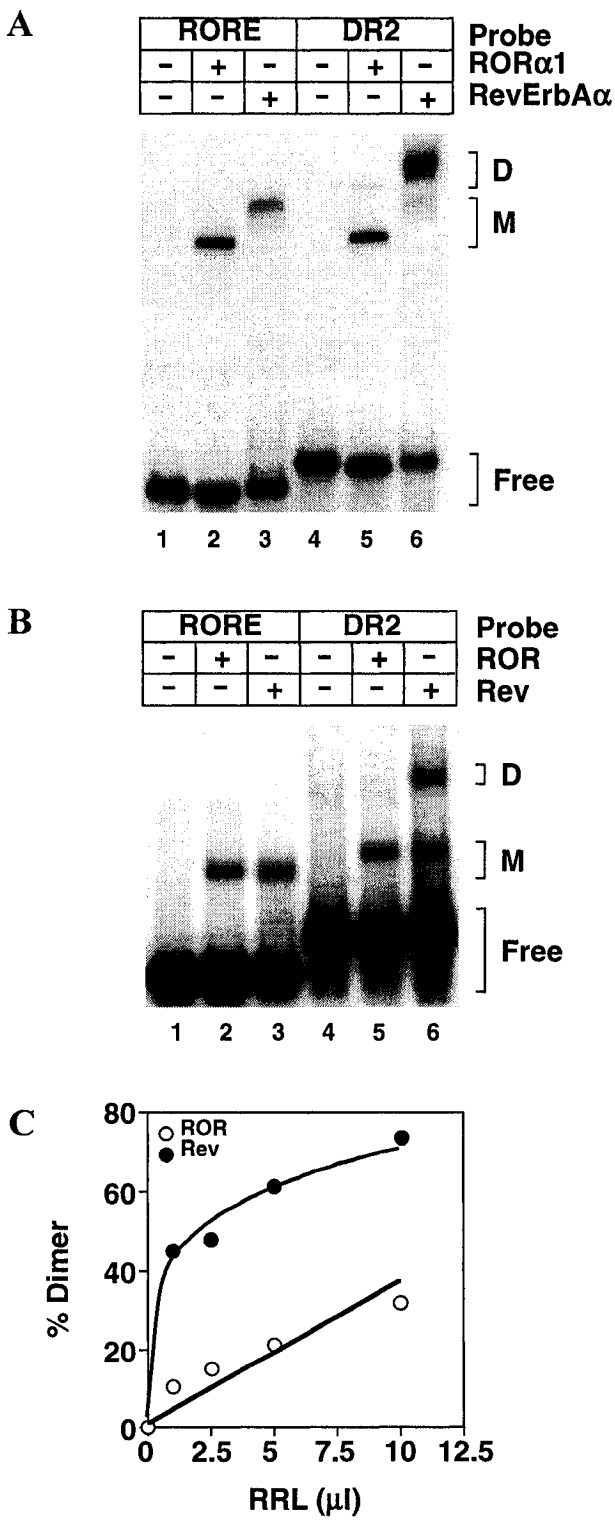
A
RORE
DR2
Probe
RORα1
RevErbAα
D
M
Free
1 2 3 4 5 6
B
RORE
DR2
Probe
ROR
Rev
D
M
Free
1 2 3 4 5 6
C
ROR
Rev
% Dimer
0
20
40
60
80
0 2.5 5 7.5 10 12.5
RRL (µl)

Figure 2. RORα is a monomeric binding orphan nuclear receptor. EMSA analysis using the RORE and extended DR2 response elements and *in vitro* translated full length RORα1 and Rev-ErbAα receptors (A) and DBD peptides (ROR and Rev) (B). (C) Quantification of dimer and monomer complexes bound to an extended DR2 element for increasing concentrations of ROR and Rev DBDs programmed rabbit reticulocyte lysate (RRL). Homodimer (D), monomer (M), unbound probe (free).

Figure 3

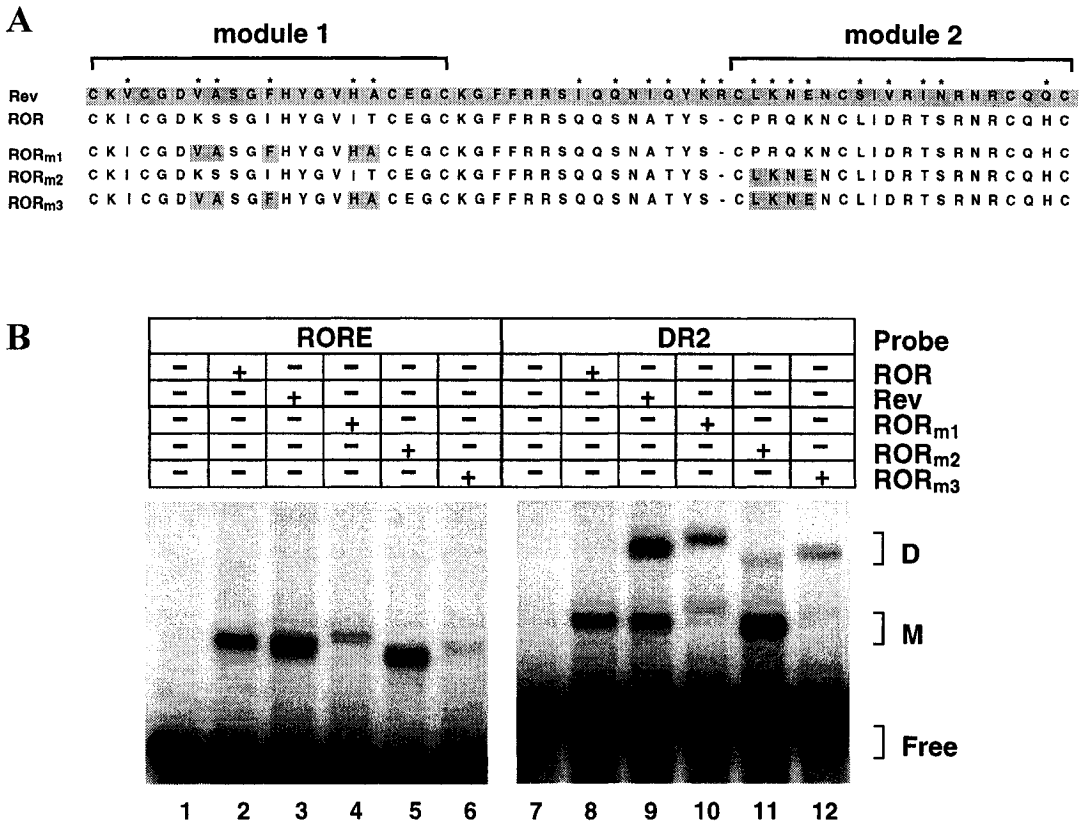
A
module 1
module 2
Rev C K V C G D V A S G F H Y G V H A C E G C K G F F R R S I Q Q N I Q Y K R C L K N E N C S I V R I N R N R C Q Q C
ROR C K I C G D K S S G I H Y G V I T C E G C K G F F R R S Q Q S N A T Y S - C P R Q K N C L I D R T S R N R C Q H C
RORm1 C K I C G D V A S G F H Y G V H A C E G C K G F F R R S Q Q S N A T Y S - C P R Q K N C L I D R T S R N R C Q H C
RORm2 C K I C G D K S S G I H Y G V I T C E G C K G F F R R S Q Q S N A T Y S - C L K N E N C L I D R T S R N R C Q H C
RORm3 C K I C G D V A S G F H Y G V H A C E G C K G F F R R S Q Q S N A T Y S - C L K N E N C L I D R T S R N R C Q H C
B
RORE
DR2
Probe
ROR
Rev
RORm1
RORm2
RORm3
D
M
Free
1 2 3 4 5 6 7 8 9 10 11 12
C
%Dimer
80
60
40
20
0
ROR Rev RORm1 RORm2 RORm3

Figure 3. RORα DBD peptide encoding Rev-ErbAα's zinc finger module 1 homodimerizes on an extended DR2. (A) The primary sequences of the DBD core comprising the two zinc finger modules beginning with the first cysteine of the first zinc finger as well as those of chimeric ROR/Rev constructs are shown. Asterisks indicate the amino acids that are not conserved between RORα and Rev-ErbAα. (B) EMSA analysis of *in vitro* translated ROR, Rev, and ROR DBD mutants using RORE and an extended DR2 as probes. (C) Quantification of dimer and monomer complexes formed by ROR, Rev and ROR mutants on an extended DR2 element. Results are presented as fraction (%) of probe bound by receptor dimers formed on an extended DR2 element.

Figure 4

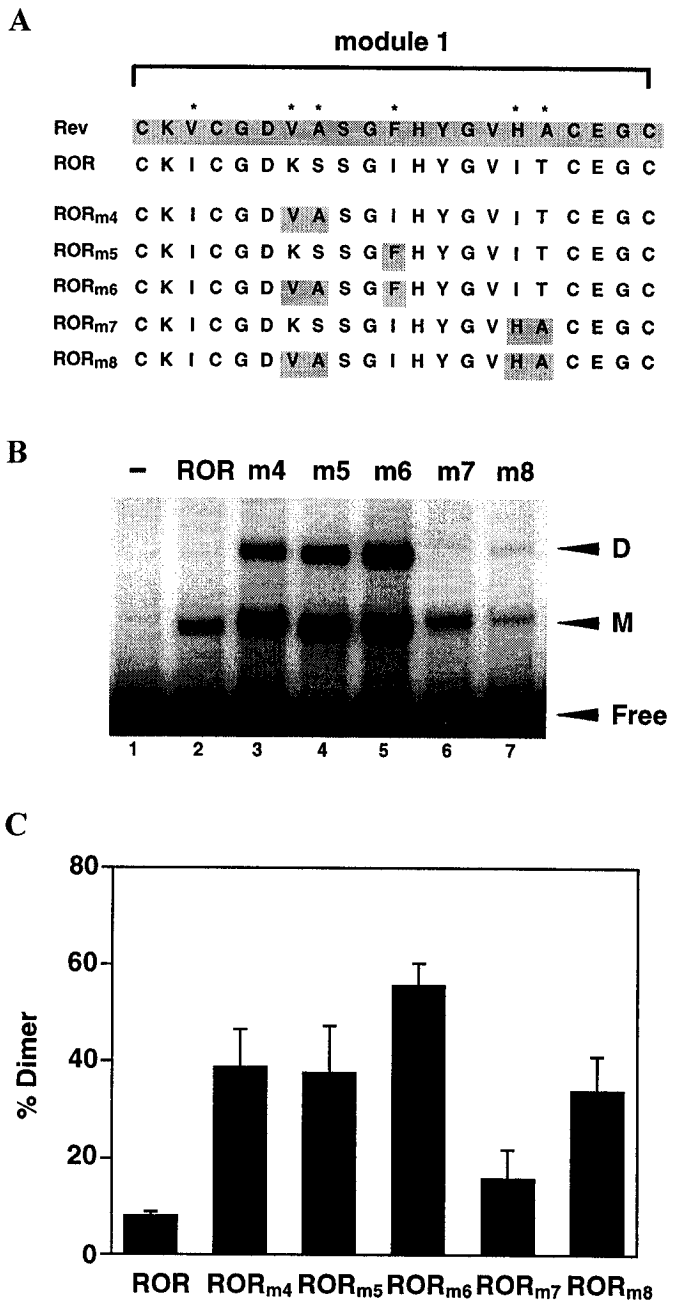
A
module 1
Rev C K V C G D V A S G F H Y G V H A C E G C
ROR C K I C G D K S S G I H Y G V I T C E G C
RORm4 C K I C G D V A S G I H Y G V I T C E G C
RORm5 C K I C G D K S S G F H Y G V I T C E G C
RORm6 C K I C G D V A S G F H Y G V I T C E G C
RORm7 C K I C G D K S S G I H Y G V H A C E G C
RORm8 C K I C G D V A S G I H Y G V H A C E G C
B
– ROR m4 m5 m6 m7 m8
D
M
Free
1 2 3 4 5 6 7
C
% Dimer
80
60
40
20
0
ROR RORm4 RORm5 RORm6 RORm7 RORm8

Figure 4. Three amino acids in Rev-ErbAα's zinc finger module 1 are sufficient to provide RORα DBD the ability to form homodimers

(A) The primary sequences of the first zinc finger module beginning with the first cysteine of the first zinc finger as well as those of chimeric ROR/Rev DBD constructs are shown. (B) EMSA analysis of *in vitro* translated ROR DBD wild type and mutants using an extended DR2 probe (C) Quantification of the amount of ROR DBD dimer complexes.

Figure 5

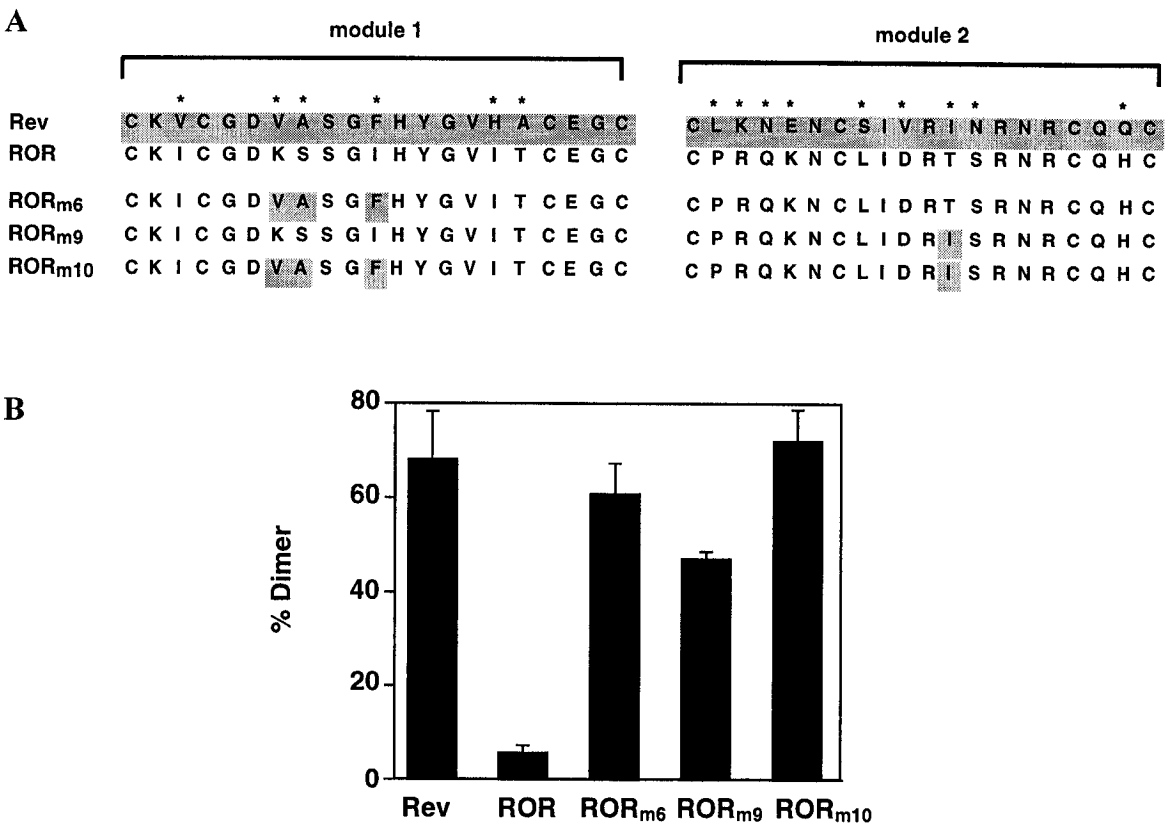
A
module 1
module 2
Rev C K V C G D V A S G F H Y G V H A C E G C C L K N E N C S I V R I N R N R C Q Q C
ROR C K I C G D K S S G I H Y G V I T C E G C C P R Q K N C L I D R T S R N R C Q H C
ROR$_{m6}$ C K I C G D V A S G F H Y G V I T C E G C C P R Q K N C L I D R T S R N R C Q H C
ROR$_{m9}$ C K I C G D K S S G I H Y G V I T C E G C C P R Q K N C L I D R I S R N R C Q H C
ROR$_{m10}$ C K I C G D V A S G F H Y G V I T C E G C C P R Q K N C L I D R I S R N R C Q H C
B
% Dimer
80
60
40
20
0
Rev
ROR
ROR$_{m6}$
ROR$_{m9}$
ROR$_{m10}$

Figure 5. Four amino acids are the key dimerization determinants. (A) Amino acid sequences of the two zinc finger modules of Rev-ErbAα, RORα, and chimera and their respective fraction (%) of probe bound by receptor dimers formed on an extended DR2 element (B).

Figure 6

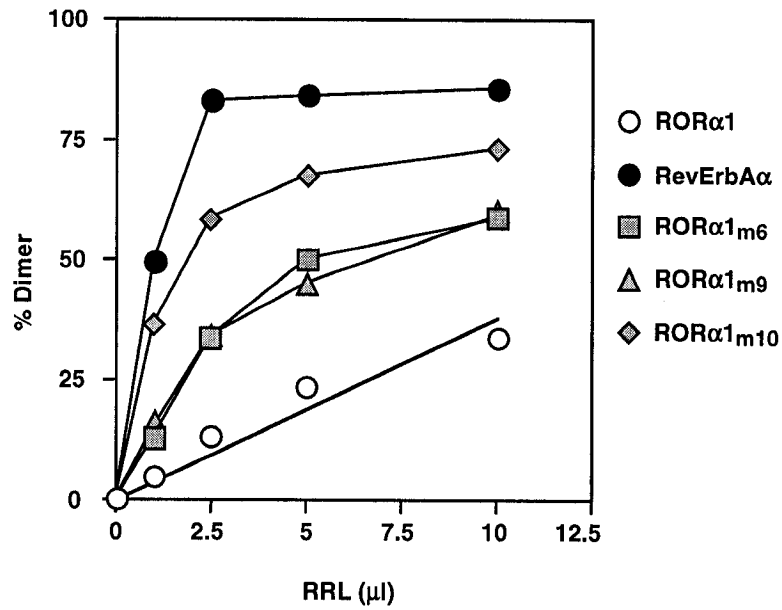
100
75
50
25
0
% Dimer
0
2.5
5
7.5
10
12.5
RRL (µl)
RORα1
RevErbAα
RORα1m6
RORα1m9
RORα1m10

Figure 6. Full length RORα1 encoding the 4 dimerization determinants forms cooperative homodimers on an extended DR2 element. The fraction (%) of probe bound by receptor dimers formed on an extended DR2 element was determined for increasing concentrations of receptor protein synthesized in programmed rabbit reticulocyte lysate (RRL) for RORα1 (white circles), Rev-ErbAα (black circles), RORα1m6 (grey squares), RORα1m9 (grey triangles), and RORα1m10 (grey diamonds).

Figure 7

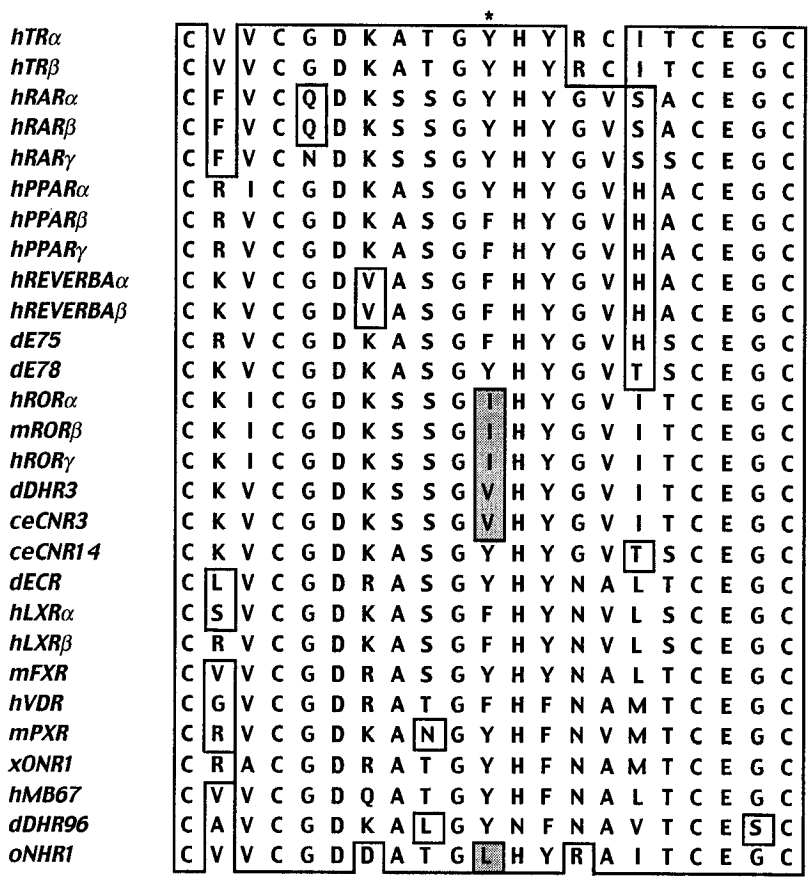
*
hTRα C V V C G D K A T G Y H Y R C I T C E G C
hTRβ C V V C G D K A T G Y H Y R C I T C E G C
hRARα C F V C Q D K S S G Y H Y G V S A C E G C
hRARβ C F V C Q D K S S G Y H Y G V S A C E G C
hRARγ C F V C N D K S S G Y H Y G V S S C E G C
hPPARα C R I C G D K A S G Y H Y G V H A C E G C
hPPARβ C R V C G D K A S G F H Y G V H A C E G C
hPPARγ C R V C G D K A S G F H Y G V H A C E G C
hREVERBAα C K V C G D V A S G F H Y G V H A C E G C
hREVERBAβ C K V C G D V A S G F H Y G V H A C E G C
dE75 C R V C G D K A S G F H Y G V H S C E G C
dE78 C K V C G D K A S G Y H Y G V T S C E G C
hRORα C K I C G D K S S G I H Y G V I T C E G C
mRORβ C K I C G D K S S G I H Y G V I T C E G C
hRORγ C K I C G D K S S G I H Y G V I T C E G C
dDHR3 C K V C G D K S S G V H Y G V I T C E G C
ceCNR3 C K V C G D K S S G V H Y G V I T C E G C
ceCNR14 C K V C G D K A S G Y H Y G V T S C E G C
dECR C L V C G D R A S G Y H Y N A L T C E G C
hLXRα C S V C G D K A S G F H Y N V L S C E G C
hLXRβ C R V C G D K A S G F H Y N V L S C E G C
mFXR C V V C G D R A S G Y H Y N A L T C E G C
hVDR C G V C G D R A T G F H F N A M T C E G C
mPXR C R V C G D K A N G Y H F N V M T C E G C
xONR1 C R A C G D R A T G Y H F N A M T C E G C
hMB67 C V V C G D Q A T G Y H F N A L T C E G C
dDHR96 C A V C G D K A L G Y N F N A V T C E S C
oNHR1 C V V C G D D A T G L H Y R A I T C E G C

Figure 7. First zinc finger module sequence alignment of group 1 nuclear receptors. The amino acid residues with a hydrocarbon sidechain in place of an aromatic ring at the position corresponding to residue 88 in RORα1 (marked by an asterisk) are highlighted.

Chapter III. Novel Mechanism of Nuclear Receptor Corepressor Interaction Dictated by AF-2 Helix Determinants

Preface

RORα is not only the prototype for monomeric DNA binding proteins, but also represents a constitutive transcriptional activator. The focus of this chapter is the molecular mechanism involved in regulating its potent transcriptional activity. We addressed whether RORa constitutive activity is dependent on an endogenous ligand and/or coactivator interaction by mutagenesis analysis of the ligand binding pocket, the hydrophobic cleft, and the AF-2. Mutagenesis of conserved amino acids in the ligand binding pocket impaired RORα activity, supporting an endogenous-ligand model. The integrity of the hydrophobic cleft and the AF-2 helix was imperative for RORα activity, and was required for the binding of SRC coactivator family members.

We also describe the molecular mechanism involved in repression of RORα activity. We observed that the Hairless (Hr) corepressor is a potent repressor of RORα-mediated transcriptional activity. In contrast to other corepressor:nuclear receptor interactions, Hr binding to RORα is mediated by two LxxLL-containing motifs, a mechanism associated with coactivator interaction. The specificity of Hr corepressor action is mediated by the AF-2 helix, in a ligand-oblivious fashion.

Abstract

Transcriptional regulation by nuclear receptors is controlled by the concerted action of coactivator and corepressor proteins. The product of the thyroid hormone-regulated mammalian gene *hairless* (Hr) was recently shown to function as a thyroid hormone receptor corepressor. Here we report that Hr acts as a potent repressor of transcriptional activation by RORα, an orphan nuclear receptor essential for cerebellar development. In contrast to other corepressor:nuclear receptor interactions, Hr binding to RORα is mediated by two LxxLL-containing motifs, a mechanism associated with coactivator interaction. Mutagenesis of conserved amino acids in the ligand binding domain indicates that RORα activity is ligand-dependent, suggesting that corepressor activity is maintained in the presence of ligand. Despite similar recognition helices shared with coactivators, Hr does not compete for the same molecular determinants at the surface of RORα ligand binding domain, indicating that Hr-mediated repression is not simply through competition for coactivator binding. Remarkably, the specificity of Hr corepressor action can be transferred to a retinoic acid receptor by exchanging the AF-2 helix. Repression of the chimeric receptor is observed in the presence of retinoic acid, demonstrating that in this context, Hr is indeed a ligand-oblivious nuclear receptor corepressor. These results suggest a novel molecular mechanism for corepressor action and demonstrate that the AF-2 helix can play a dynamic role in controlling corepressor as well as coactivator interactions. The interaction of Hr with RORα provides direct evidence for the convergence of thyroid hormone and RORα-mediated pathways in cerebellar development.

Introduction

Nuclear receptors are transcription factors that control essential developmental and physiological pathways (Mangelsdorf et al., 1995). The nuclear receptor superfamily consists of receptors that bind steroid hormones (such as estradiol and cortisone), nonsteroidal ligands (such as retinoic acid and thyroid hormone), diverse products of lipid metabolism (such as fatty acids and bile acids), as well a large group of receptors whose discoveries have preceded that of their ligands, known as orphan receptors (Giguère, 1999). Members of this superfamily control the expression of their target genes in a ligand-regulated fashion through interaction with coregulator proteins (Glass and Rosenfeld, 2000). Coregulators and associated cofactors can either repress or activate gene transcription through the recruitment of diverse functional domains and enzymatic activities to the promoters of target genes (McKenna et al., 1999). Corepressor and coactivator binding to nuclear receptors is thought to be mutually exclusive and regulated by ligand binding, making coregulator exchange a key feature in transcriptional functions of nuclear receptors (Glass and Rosenfeld, 2000).

The ligand-binding domain (LBD) of nuclear receptors mediates the ligand-dependent transactivation function through activation function 2 (AF-2), which serves as a binding surface for a diverse set of coactivators (Feng et al., 1998). AF-2 is comprised of a hydrophobic cleft formed by three (H3, H5, and H6) of the 11 helices constituting the LBD and a short amphipatic α-helix referred to as the AF-2 helix (Danielian et al., 1992). AF-2-dependent coactivators encode one or more signature

motifs of a consensus sequence LxxLL (where L is a leucine and x is any amino acid) which also form amphipatic α-helices (Heery et al., 1997). The LxxLL helix fits into the hydrophobic cleft of a liganded receptor and this interaction is stabilized by the presence of the AF-2 helix (Nolte et al., 1998; Shiau et al., 1998; Westin et al., 1998). Receptor-specific utilization of LxxLL-containing motifs is dictated by adjacent amino acid residues (Darimont et al., 1998; Mak et al., 1999; McInerney et al., 1998), and peptides containing such motifs have been shown to antagonize the activity of nuclear receptors with great specificity (Chang et al., 1999; Norris et al., 1999).

Corepressors such as N-CoR and SMRT have an autonomous repression domain and interact with unliganded non-steroid receptors (Chen and Evans, 1995; Crawford et al., 1998; Harding et al., 1997; Horlein et al., 1995; Kurokawa et al., 1995; Lee et al., 1995; Sande and Privalsky, 1996; Shibata et al., 1997; Zamir et al., 1996), as well as to antagonist-bound steroid receptors (Jackson et al., 1997; Lavinsky et al., 1998; Smith et al., 1997). Like coactivators, these proteins encode an extended amphipatic helix whose sequence contains the residues ΦxxΦΦ (where Φ is a hydrophobic residue and x is any amino acid) (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). In a manner analogous to the LxxLL-containing motifs, mutational analysis has suggested that this extended helix also makes contacts with residues in the hydrophobic pocket but is not dependent on the charged clamp and the AF-2 helix (Nagy et al., 1999; Perissi et al., 1999). Indeed, deletion of the AF-2 helix enhances corepressor binding (Chen and Evans, 1995), suggesting that the helix does not play an active role in nuclear receptor:corepressor recognition.

RORα (Retinoic acid receptor related Orphan Receptor α) {NR1F1} is a constitutively active orphan nuclear receptor that plays a vital role in cerebellar development, lipid metabolism and neoplasia (reviewed in Dussault et al., 1998; Giguère, 1999; Matysiak-Scholze and Nehls, 1997; Steinmayr et al., 1998) and RVR {NR1D2} orphan nuclear receptors which lack an AF-2 helix (Dussault and Giguère, 1997; Forman et al., 1994; Retnakaran et al., 1994). Repression of ROR-regulated gene expression may be functionally significant as generation of a null mutation in the gene encoding Rev-ErbAα results in delayed Purkinje cell differentiation, suggesting that inhibiting the expression of RORα-induced genes is required for maturation of these cells (Chomez et al., 2000). A third factor known to be important for cerebellar development is thyroid hormone (T_3). T_3 deficiency affects a number of developmental processes in neonatal cerebellum including cell migration, differentiation and synaptogenesis (Koibuchi and Chin, 2000). Thus, cerebellar development is likely to be regulated through the cross-talk of T_3R, RORα and Rev-ErbAα nuclear receptors.

A search for T_3-regulated genes in the cerebellum resulted in the isolation of the rat *hairless* (*hr*) gene (Thompson, 1996). *Hr* is expressed at high levels shortly after birth and is a direct target gene of T_3, as it has a potent T_3 response element and is rapidly induced even in the absence of protein synthesis (Thompson, 1996; Thompson and Potter, 2000). Multiple mutant *hr* alleles have been described that result in the *hairless* phenotype both in mice (Stoye et al., 1988) and in humans (Ahmad et al., 1998; Cichon et al., 1998). The *hr* gene product (Hr) has been shown to be a corepressor that mediates transcriptional repression by unliganded T_3R (Potter

et al., 2001; Thompson and Bottcher, 1997). Hr interacts with histone deacetylases (HDACs) and localizes to matrix-associated deacetylase (MAD) bodies, indicating that the mechanism of Hr-mediated repression is similar to other corepressors (Potter et al., 2001).

Given the potential cross-talk between T_3R and RORα in cerebellar development, we investigated whether Hr was a common cofactor of these regulatory pathways. Here we show that Hr is a potent repressor of RORα transcriptional activity, and that the specificity of the interaction between Hr and RORα is dictated by the primary structure of the AF-2 helix. These results define a novel role for the AF-2 helix in corepressor/nuclear receptor interactions and suggest that Hr, RORα and T_3R belong to a common ligand-based developmental regulatory network.

Materials and Methods

Yeast two hybrid assay. The yeast two hybrid assay was performed as previously described (Hollenberg et al., 1995; Thompson and Bottcher, 1997). pLexA-Hr568-1207, pLexA Hr 782-1207 and pLexA-Hr 568-784 have been described (Potter et al., 2001; Thompson and Bottcher, 1997). pVP16-RORα was constructed by excising the RORα LBD from pCMXGAL4hRORα_{LBD} by digestion with *Eco*RI and *Bam*HI and inserting the fragment into the *Eco*RI-*Bam*HI sites of pVP16 (Hollenberg et al., 1995).

Plasmid Construction. pCMX-VP16hRORα1 was made as follows: pCMX-hRORα1 described elsewhere (Giguère et al., 1994) was digested with *Not*I/*Bam*HI restriction enzymes yielding a 1.7 kb fragment (including amino acids 22-523), cloned into the *Not*I/*Bam*HI sites of pCMXVP16$_N$ containing a *Not*I linker. pCMX-Flag-hRORα1 was made by introducing by PCR *Eco*RI and *Bam*HI sites at the 5' and 3' ends respectively of RORα (aa 1-523) and cloning into pCMX-FLAG vector. pCMXGAL4hRORα_{LBD} encoding amino acids 270-523 was constructed by cloning in frame a *Eco*RV/*Bam*HI fragment from pCMXhRORα1 downstream of the GAL4 DBD sequence. pKShROR$\alpha 1_{LBD}$ was constructed by cloning the same *Eco*RV/*Bam*HI fragment into BluescriptKS$_{II}$ (Stratagene, La Jolla, CA). pKS-RORα_{LBD} was used as a template for site-directed mutagenesis generating the following LBD mutants: C288F, W320A, C323A, E329A, A330T, V335R, K339A, I353A, K357A, L361F, V364G, F365Y, M368A, A371G, Y380A, D382V, G395D, F399Y, H484A, L488A, F491A, F503A, L506R, Y507A, E509K and L510A. Mutations were verified by sequencing

followed by subcloning of the *Eco*RV-*Bam*HI fragment into the pCMX-hRORα1 backbone. pCMX-RORαΔAF2 was generated by mutating E509A, L510A, F511A, T512A residues of helix 12. pCMX-RORαV335R/ΔAF2, K339A/ΔAF2, I353A/ΔAF2 AND K357A/ΔAF2 were generated by sucloning a 509bp *Xba*I/*Bam*HI fragment encoding the mutated H12 into the pCMX-RORα cleft mutant backdone.

The mouse RORβ and RORγ cDNAs were isolated from a brain and skeletal muscle λgt11 cDNA library (Clonetech), respectively. Both pCMXmRORβ and pCMXmRORγ were generated by subcloning *Eco*RI fragments containing the full length cDNAs for both RORβ and RORγ respectively into pCMX expression vector.

pRK5-mycrhr has been described elsewhere (Potter et al., 2001). pRK5mycrHr was used as a template for site directed mutagenesis using *Pfu* polymerase (Stratagene, La Jolla, CA) generating Hr_{m1} (L586A), Hr_{m2} (L589A, L590A), Hr_{m3} (L781A, L782A), Hr_{m4} (I820A, I821A), Hr_{m5} (L589A, L590A, L781A, L782A), Hr_{m6} (L586A, L781A, L782A) Hr_{m7} (L589A, L591A, I820A, I821A), Hr_{m8} (L781A, L782A, I820A, I821A). These and all subsequent mutations were verified by sequencing. To generate pCMXGAL4-$Hr_{568-1207}$, a 2.21 kb *Hind*III fragment from rat Hr was blunted using Klenow, *Bam*HI linkers were added and ligated into the *Bam*HI site pCMXGAL4. pCMXGAL4-$Hr_{568-784}$ was constructed by digesting pCMXGAL4-$Hr_{568-1207}$ with *Nhe*I, isolating the vector fragment and re-ligating, resulting in the deletion of the Hr sequences downstream of the *Nhe*I site at position 2732 of the cDNA. pCMX-Hr^{RID} encompassing amino acids 568-784 was generated by adding by PCR, *Asp*718 and *Bam*HI restriction sites at the 5' and 3' ends of this region respectively, followed by subcloning into the pCMX backbone.

pCMXhRARα and pCMXhRXRα were described elsewhere (Umesono et al., 1991). pCMXhRARα-R was constructed by site directed mutagenesis using *Pfu* polymerase of pCMXhRARα template, introducing a 5 amino acid change in the AF-2 helix: I410Y, Q411K, M413L, L414F, E414T. These were verified by sequencing, followed by subcloning of a 286 bp *Sma*I fragment encoding the mutations into the pCMXhRARα backbone. Reporter constructs RORE$\alpha 2_3$TKLuc, UAS_2TKLuc, TREpal_3TKLuc were previously described (Giguère et al., 1994; Tini et al., 1995). pCMX-HA-RARα-R and pCMX-HA-RARα were constructed using the following method. HA tag (CYPYDVPDYASLEF) annealed oligonucleotides flanked by *Cla*I and *Eco*RI at the 5' and 3' end respectively were cloned between the *Cla*I/*Eco*RI sites of pCMX, yielding pCMX-HA. Amino acids 2-462 of pCMX-hRARα and pCMXhRARα-R was amplified by PCR. *Eco*RI and *Bam*HI sites were introduced at the 5' and 3' ends respectively, followed by subcloning into the pCMX-HA vector.

The receptor interacting domains (RID) of the SRC family members were amplified by PCR using *Pfu* polymerase and oligonucleotides that introduce a *Bam*HI and an *Eco*RI site on the 5' and 3' ends respectively, followed by subcloning into the *Bam*HI/*Eco*RI sites of the pGEX2T vector. pGEX2TmSRC1a_{RID} includes amino acids 565-787, pGex2TmGRIP1_{RID} includes amino acids 563-767, and pGEX2Tmp/CIP_{RID} includes amino acids 547-785. pRK5myc-rHr and pRK5myc-$rhr_{m1\text{-}m8}$ were digested with *Hind*III and *Sac*I restriction enzymes generating a 891bp fragment, encoding amino acids 568-864, blunted using Klenow an ligated into pGEX2T vector digested by *Sma*I, yielding pGEX2T-$rHr_{568\text{-}784}$ and pGEX2T-$rHr_{m1\text{-}m8}$. The RID of SMRT

(1080-1495) was amplified by PCR and cloned into the *Bam*HI-*Eco*RI site of pGEX-2T vector, yielding pGEX2T-SMRT$_{RID}$ (provided by M. Latreille, McGill University).

Protein Expression and GST Pull-Down Assays. The various bait constructs were transformed in E. coli DH5α. GSTSRC1a$_{RID}$, GST-GRIP1$_{RID}$, GST-P/CIP$_{RID}$, GST-Hr, GST-Hr$_{m1-m8}$, and GST-SMRT$_{RID}$ protein expression was induced with 0.5 mM isopropylthiogalactopyranoside (IPTG) at 37°C for 3hrs. Bacterial extracts were prepared by sonication in a 1% Triton-X phosphate buffered saline solution. The amount of bacterial extract used in each experiment was determined based on a coomassie stained 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) used to compare for equal protein expressed. The bacterial extracts were bound to 30 μl of a 50% slurry of glutathione-Sepharose beads (Pharmacia Biotech) in NET-N buffer (150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl (pH 8.0), 1.0% TritonX-100, 1μ M leupeptin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) for 30 min of mild rotation at 4°C. The beads were then washed twice in GST-binding buffer (20 mM HEPES (pH7.9), 150 mM KCl, 0.1% 3-{(3-Cholamidopropyl)dimethyl-ammonio}-1-propanesulfonate (CHAPS), 20 μl/ml BSA, 0.1 mM PMSF and 1 mM leupeptin). 5 μl of in vitro translated ^{35}S-methionine labeled proteins, using T$_N$T rabbit reticulocyte lysate (Promega, Madison, WI), were added to the beads in a final volume of 150 μl of GST-binding buffer and incubated for 1 hr 30 min at 4°C with mild rotation. The complexes were washed twice in GST-binding buffer. They were then resuspended in 1x SDS-sample buffer and boiled for 5 min prior to loading on a 10% SDS-PAGE. The gels were fixed in 25% isopropanol/10% acetic acid, followed

by treatment with the flurographic reagent Amplify (Amersham Life Science), dried and exposed.

Cell Culture and Transient Transfection. Cos-1 cells obtained from the American Type Culture Collection were cultured in Dulbecco's Minimal Essential Medium (DMEM) containing penicillin (25 U/ml), streptomycin (25 U/ml) and 10% fetal calf serum at 37°C with 5% CO_2. Twenty-four hours prior to transfection the cells were split and seeded in 12 well-plates. The cells were transfected with FuGENE 6 Transfection Reagent (Roche Diagnostics), following protocol supplied by the manufacturer, and harvested 24 hrs after transfection. Typically, 0.05 μg of receptor plasmid, 0.5 μg of pRK5-mycrhr, 0.5 μg of reporter plasmid, and 0.25 μg of internal control pCMVβGal were transfected per well. For the mammalian two-hybrid assay 0.2 μg pCMXVP16hRORα1, 0.01 μg pCMX-GAL4-rHr, 0.5 μg pCMX-UAS_{2c}TKLuc, 0.25 μg pCMVβGal and BluescriptKS plasmid to a total of 1 μg DNA per well. For transfection of RARα/RARα-R, the cells were seeded in DMEM supplemented with 10% charcoal-dextran treated fetal calf serum 24 hrs prior to transfection. Four hours after transfection, the cells were washed twice with 1x PBS and fresh media was added containing ethanol (vehicle) or all-trans retinoic acid (at-RA) to final concentration of 10^{-8} M. Cells were then harvested 16 hrs later and assayed for luciferase and β-galactosidase. 0.05 μg pCMXhRARα/hRARα-R and pCMXhRXRα, 0.25 μg pRK5-mycrhr, 0.5 μg $TREpal_3$TKLuc, 0.25 μg pCMVβGal were transfected per well.

Immunoprecipitation and Western Blotting. Cos-1 cells were transiently transfected with 5μg of pCMX-FlagRORα, pCMX-HAhRARα, pCMX-HAhRARα-

RpRK5-mycrHr as described above. Cells were lysed in IP buffer (1%NP-40, 10% glycerol, 150mM NaCl, 50mM Tris-HCl pH 7.5) supplemented with protease inhibitor cocktail (Complete Mini EDTA-free, Roche Diagnostics). Lysates were incubated with either Flag antibody (Sigma), HA antibody (Upstate Biotechnology) or Hr antibody (MD9-Hr) overnight at 4°C, with gentle rotation. Proteins were collected on either protein A- or protein G-Sepharose for 3hrs at 4°C with mild rotation and then washed three times with low salt buffer (1%NP-40, 50mM Tris-Hcl pH8.0). Imunoprecipitates were resolved by SDS-PAGE, and transferred to a nitrocellulose membrane and immunoblotted with Flag antibody, HA antibody (Covance) or Hr antibody Proteins were visualized with the POD chemiluminescence kit following manufacturer's instructions (Roche Diagnostics). Immunoblotting for detection of Hr mutant proteins was similarly done using lysates from transiently transfected Cos-1 cells and immunoblotting with Hr antibody.

Results

RORα shares functional and structural determinants with classic nuclear receptors. The amino acid residues involved in forming the hydrophobic cleft required for coactivator interaction are highly conserved among members of the nuclear receptor superfamily. Formation of this hydrophobic cleft is also dependent on the AF-2 helix. Recently, resolution of the crystal structure of RORβ LBD demonstrated that the members of the ROR family share the same canonical fold described for other nuclear receptors, with an additional 2 α-helices (Stehlin et al., 2001). The presence of a functional ligand binding pocket (LBP), a hydrophobic cleft and AF-2 helix at the surface of RORβ LBD is maintained. We first used site directed mutagenesis to assess the involvement of these determinants in RORα constitutive transcriptional activity and their interaction with the three members of the SRC family of coactivators. Residues were targeted according to previous functional analyses of nuclear receptor/coactivator interaction demonstrating the importance of specific conserved residues in these interactions (Fig. 1A). As shown in Fig. 1B, mutation of residues participating in the formation of the hydrophobic cleft resulted in complete loss of RORα transcriptional activity when assayed by transient transfection with a reporter plasmid consisting of the monomeric RORE linked to the basal TK promoter. The loss of RORα transcriptional activity is correlated with loss of interaction with members of the SRC family of coregulators as measured in a GST pull-down assay (Fig. 1C). These results extend observations previously made using mutant Gal4DBD-RORα chimeras and GRIP-1 (Atkins et al., 1999) to the native

RORα and all members of the SRC family. RORα differs from other nuclear receptors with respect to the importance of K357 in H4. This residue has been shown to be required for the formation of a functional coactivator surface (Feng et al., 1998). Mutation of K357A does not affect RORα transcriptional activity (Fig. 1B) and interaction with SRC family members remains unhindered (Fig. 1C). This is in agreement with data provided by the RORβ crystal structure in which this residue was not shown to make contact with SRC LxxLL helix.

X-ray structure analyses complemented by extensive mutational studies of nuclear receptor LBDs have defined the determinants required for high affinity ligand binding (reviewed in Wurtz et al., 1996). By analogy with data derived from analysis of RARγ and RORβ, we have generated a set of RORα mutants carrying point mutations that, in the context of RARγ and RORβ, either abolish or significantly diminish the ability to recognize their cognate ligands, thus hampering their ability to transactivate (Fig. 1A). As seen in Fig. 1D, for 12 of 19 mutant receptors transcriptional activity was diminished by more than 50%. All mutant receptors were expressed at similar levels as measured by Western blot analysis (data not shown). These results strongly suggest that the transcriptional activity of RORα is regulated by a ligand present endogenously in cultured cells. This data also lends support to the differences within the ligand binding pocket (LBP) of ROR family members. Particularly, residues A330, L361 and F399, are required for ligand binding for both RORβ and RARγ (Fig. 1A), but are not required for ligand binding by RORα leading to transactivation levels equivalent to wild type (Fig. 1D). In general, RORα, RORβ and RORγ likely share the

same overall structure but significant differences within the LBP would allow each receptor to discriminate their respective ligands.

Hr is a repressor of orphan nuclear receptor RORα. Hr is a newly identified nuclear receptor corepressor that has been shown to interact specifically with T_3R (Potter et al., 2001; Thompson and Bottcher, 1997). While the Hr protein does not share sequence identity with previously characterized nuclear receptor corepressors, it encodes four nuclear receptor interaction motifs (Fig. 2A). Two motifs have the coactivator LxxLL-containing consensus sequence and two include the sequence ΦxxΦΦ thought to mediate corepressor interaction. Since RORα and T_3R may be part of a common regulatory pathway controlling cerebellar development, we investigated whether Hr could also modulate RORα transcriptional activity. As shown in Fig. 2B, co-expression of Hr and RORα leads to nearly complete inhibition of the potent constitutive transcriptional activity displayed by RORα. Given the high degree of identity and functional similarity between members of the ROR family (Giguère, 1999), we next tested whether Hr could inhibit the activity of the RORβ and γ isoforms. Hr antagonizes the transcriptional activity of RORβ and RORγ as well (Fig. 3B), indicating that Hr is a corepressor of all ROR isoforms and that Hr interaction determinants are likely conserved within the family.

The presence of nuclear receptor interaction motifs within Hr and the ability to repress transcriptional by all ROR isoforms indicated that Hr might interact with the ROR LBD. To assess this possibility, we first generated a chimeric protein in which the DNA binding domain of the yeast Gal4 transcription factor was linked to the LBD of RORα (Fig. 2C). When transiently expressed in Cos-1 cells with a Gal4UASLuc

reporter plasmid, the Gal4-RORα^{LBD} chimera displays constitutive transcriptional activity as potent as the activity generated by the native receptor. Similarly, the transcriptional activity of the Gal4-RORα^{LBD} chimera is completely abolished by Hr, demonstrating that repression is mediated through the LBD and is independent of the reporter gene used in the assay.

We next tested whether the region of Hr encoding the nuclear receptor interaction motifs was sufficient to promote Hr/RORα interaction. Fig. 3A depicts the result of a yeast two-hybrid experiment in which fragments of Hr were fused with the LexA DBD and the activation function of VP16 was fused to RORα. Both the carboxy-terminal fragment (Hr 568-1207) and an internal fragment (568-784) interact with RORα. Surprisingly, $Hr^{568-784}$ contains the two coactivator interaction LxxLL motifs, while the non-interacting fragment (782-1207) contains the two corepressor motifs previously shown to mediate interaction with T_3R (Potter et al., 2001). Analysis of Hr/ROR interaction in a mammalian two-hybrid experiment gave similar results (Fig. 3B). Fragments of the Hr protein were fused to the Gal4 DBD while the activation function of VP16 was fused to RORα. The resulting constructs were cotransfected in Cos-1 cells together with a Gal4 UAS reporter and interaction was measured by luciferase assay. As shown, both the carboxy-terminal Hr fragment (568-1207) and the smaller internal fragment (568-784) interact with RORα in mammalian cells. These results indicate that it may be the coactivator binding motifs and not the corepressor interaction motifs that play a role in Hr/RORα interaction.

Direct interaction between RORα and Hr was tested using GST pull-down experiments. As shown in Fig. 3C, native RORα interacts very weakly with Hr but

strongly with SRC-1. However, it has been observed that interaction between nuclear receptors and corepressors such as SMRT and N-CoR is enhanced upon inactivation of the AF-2 helix (Chen and Evans, 1995). We thus generated an AF-2 helix-deficient form of RORα and tested its ability to bind to Hr *in vitro*. The AF-2 helix-deficient RORα mutant displays a complete reversal in binding activity: strong interaction with Hr and a total loss in its ability to bind SRC-1. We next tested whether Hr interacts with RORα *in vivo*. As shown in Fig. 3D, Flag-tagged RORα coimmunoprecipitates with Hr in transiently transfected Cos-1 cells. Although the AF-2 helix hinders Hr binding *in vitro*, this is not the case *in vivo* where interaction between RORα and Hr occurs. This suggests that a third component required for Hr binding is missing in the *in vitro* system. One explanation for this phenomenon is that post-translational modification of RORα may influence the dynamics of the AF-2 helix promoting interaction with Hr. There are three species detected by the Flag antibody, which may represent post-translationally modified forms of RORα. A second possibility is that a third protein acting as a bridging factor is required as a ternary partner for RORα/Hr interaction.

Repression of RORα activity by Hr is dependent on two LxxLL motifs. While the above results indicate that Hr:RORα binding is mechanistically similar to that of a classic nuclear receptor:corepressor interaction, based on our deletion analysis (Fig. 3A), its interaction with RORα appears to be dictated through coactivator-like recognition motifs. To test this hypothesis, we introduced a series of point mutations in three of the nuclear receptor recognition motifs (Fig. 4A) and assayed the ability of the mutated Hr to repress RORα transcriptional activity in Cos-1 cells. All mutants

were expressed at similar levels as shown by the Western blot (Fig. 4B, lower panel). As shown in Fig. 4B (upper panel), mutations of the proximal leucine residue (Hr^{m1}) and two distal leucine residues (Hr^{m2}) in the first LxxLL motif leads to a ~50% loss in Hr repressive activity. Likewise, mutation of the two distal leucine residues in the second LxxLL motif (Hr^{m3}) also results in a sharp diminution of Hr activity. In contrast, mutations within the ΦxxΦΦ motif (Hr^{m4}) has no deleterious effect on Hr function. However, the ability of Hr to repress RORα activity was completely lost when combinations of mutations in both LxxLL were introduced in Hr (Hr^{m5} and Hr^{m6}). Combinations of mutations in either LxxLL motif together with the ΦxxΦΦ motif (Hr^{m7} and Hr^{m8}) resulted in Hr mutants with activity similar to the individual LxxLL mutants. Finally, the GST pull-down experiment shows that the levels of *in vivo* activity displayed by Hr mutants correlate well with their RORα binding activity *in vitro* (Fig. 4C). Unexpectedly, these results demonstrate that the repressive activity of Hr is dependent on the presence of the two LxxLL motifs rather than the ΦxxΦΦ motifs.

Since Hr binds to ROR via LxxLL motifs, a mechanism shared by coactivators such as SRC-1, repression of ROR activity by Hr may occur by occluding coactivator binding. To test whether Hr LxxLL motifs and SRC LxxLL motifs share the same determinants at the surface of the RORα LBD, we generated constructs containing both mutations in the hydrophobic cleft and the AF-2 helix and tested their ability to interact *in vitro* with Hr in a GST pull-down assay (Fig. 4D). Mutation of residues (V335, K339, I353) which are important for SRC-1 binding did not affect binding of Hr. This suggests that although Hr and SRC share similar recognition helices, they do

not compete for the same molecular determinants at the surface of the RORα LBD. We next used a putative dominant negative Hr construct containing only the receptor interacting domain (RID) and cotransfected in both wild type Hr and RORα. HR^{RID} did not affect RORα transcriptional activity but did hinder Hr repression. This demonstrates that Hr^{RID} indeed acts as a dominant negative for Hr action, and importantly, it does not displace endogenous coactivators.

Specificity of Hr nuclear receptor targets is conferred by the AF-2 helix. RORα is closely related to RARα, yet Hr does not bind RARα (Potter et al., 2001; Thompson and Bottcher, 1997). Given that coactivator-type binding motifs mediate ROR binding, we hypothesized that the specificity of Hr for RORα is conferred by the AF-2 helix. Previous observations that the C-terminal domain of RORβ is functional in the context of the RARα LBD (Greiner et al., 1996) suggested that a RARα/RORα chimera could constitute a useful tool to test this idea. Thus, to determine if Hr binding could be transferred to a heterologous receptor, we generated a RARα mutant receptor in which the primary amino acid sequence of the AF-2 helix was changed to that of RORα, a change of only 5 amino acids (RARα-R) (Fig. 5A). We first tested whether the RARα-R chimeric protein retained the transcriptional properties of wild type RARα. Using an *in vitro* GST pull-down assay, we showed that the RARα-R chimera is able to bind SRC-1 in a ligand-dependent fashion as well as its wild type RARα counterpart (Fig. 5B). Similarly, the RARα-R chimera interacts with SMRT in the absence of retinoic acid and this interaction is abolished by the addition of ligand (Fig. 5C). These observations not only demonstrate that the RARα-R mutant is functional but, perhaps more importantly, that the AF-2 helix of RORα

functions properly in the context of a liganded receptor, adding support to the hypothesis that RORα activity is indeed regulated by an endogenous ligand. Next, we tested the chimeric receptor for transcriptional activity. As expected, RARα activated gene transcription in response to *all-trans* retinoic acid in a transient transfection assay (Fig. 5D). This response was not affected by the presence of Hr. Strikingly, RARα-R showed retinoic acid-dependent transcriptional activity, and co-transfection of Hr dramatically decreased the transcriptional activity of RARα-R. Finally, we show that the observed repression of the modified RAR-R is due to recruitment of Hr. As shown in Fig. 5E, complex immunoprecipited with the Hr antibody contains RAR-R but not wild-type RAR. The specificity of interaction between Hr/RAR-R is further highlighted by the observation of a slight decrease in interaction between these two proteins in the presence of retinoic acid, possibly reflecting a competition between Hr and coactivator complexes. These results clearly demonstrate that the specificity of Hr interaction with nuclear receptors resides within the AF-2 helix. Furthermore, this data also shows that unlike other corepressors whose interaction with nuclear receptors is disrupted upon ligand binding (Chen and Evans, 1995; Horlein et al., 1995), Hr repression of RARα-R activity occurs in the presence of ligand. These results suggest that Hr function is unhindered by the presence of ligand in the context of the AF-2 helix of RORα, and thus Hr constitutes a distinct type of nuclear receptor corepressor.

Discussion

Nuclear receptors are transcriptional regulators capable of both activating and repressing specific gene networks in response to developmental and physiological cues. The choice between activation and repression is thought to depend on specific, mutually exclusive interactions with coactivators and corepressors. These interactions take place through common surface determinants in the receptor LBD and are tightly regulated by ligand binding (reviewed in Glass and Rosenfeld, 2000). This proposed mode of action constitutes an elegant and simple molecular mechanism through which a family of ligand-dependent transcription factors can efficiently and precisely control the expression of target genes.

The existence of constitutively active orphan nuclear receptors whose activity might be continuously stimulated by the presence of ubiquitous ligands (reviewed in Giguère, 1999) suggests that this class of nuclear receptors may utilize related but distinct molecular mechanisms to regulate their transcriptional functions. Here, we describe the functional interaction between RORα, a constitutively active orphan nuclear receptor, with a novel corepressor, the Hr protein. This study shows a novel function for Hr as a potent ligand-oblivious nuclear receptor corepressor. Strikingly, these results demonstrate that the targets of nuclear receptor corepressors can be specified by determinants encoded within the AF-2 helix.

Hr is a bifunctional corepressor. Despite its lack of sequence identity with previously described corepressors such as SMRT and N-CoR, Hr has been shown to function as a nuclear receptor corepressor (Potter et al., 2001; Thompson and

Bottcher, 1997). Hr interacts directly and specifically with T_3R, and can mediate transcriptional repression of unliganded T_3R. Interaction with T_3R is mediated by two ΦxxΦΦ-containing domains, and Hr likely mediates transcriptional repression through associated histone deacetylase activity (Potter et al., 2001). These data suggest that in the context of T_3R, Hr functions in a manner similar to SMRT and N-CoR.

The finding that Hr, the same protein that can mediate ligand-independent repression by T_3R, can also influence the activity of a constitutively active orphan receptor, indicates that Hr can serve multiple roles in mediating transcriptional repression. Evidence that RORα may bind to an as yet unknown ligand suggests that Hr interacts with ligand-bound RORα, exactly the opposite of its mechanism of action on T_3R. This assumption is clearly validated by the observation that Hr represses transcriptional activation by the retinoic acid-activated chimeric RARα-R protein (Fig. 5). Thus Hr is a bifunctional corepressor, which can interact with different classes of nuclear receptors through distinct, well-conserved interaction domains: with T_3R through ΦxxΦΦ motifs (Potter et al., 2001) and with RORα via two LxxLL motifs (Fig. 4).

The interaction of Hr with RORα through coactivator type binding motifs suggests that Hr might compete for coactivator binding. However, our results show that Hr docking onto RORα does not require the same molecular determinants on the surface of the LBD. In addition, expression of the minimal region of Hr shown to bind to RORα does not hinder transcriptional activation as would be expected if Hr interaction displaced coactivator binding. Thus, repression of ligand bound RORα by Hr is not due to mere competition or occlusion of the coactivator binding site, but

rather likely occurs through one or more of the independent repression domains previously defined in Hr (42).

The AF-2 helix dictates corepressor binding specificity. Biochemical and X-ray crystallographic studies have shown that the AF-2 helix plays a crucial role in controlling the assembly of nuclear receptors and coactivator proteins (Darimont et al., 1998; Nolte et al., 1998; Shiau et al., 1998; Westin et al., 1998). The AF-2 helix participates in the formation of a charged clamp defined by highly conserved residues among nuclear receptors, suggesting a shared structural role for the AF-2 helix in the common mechanism for coactivator binding with nuclear receptors. This study reveals for the first time that the AF-2 helix can also mediate binding between a corepressor and a nuclear receptor. Indeed, introduction of the AF-2 helix sequence of RORα within the otherwise intact RARα, a change of only five amino acids, allowed Hr to repress the transcriptional activity of the mutant RARα (Fig. 5). Thus, the primary amino acid sequence of the AF-2 helix can dictate binding specificity between a corepressor and a nuclear receptor. This observation implies that nuclear receptor AF-2 helices, although highly conserved, encode unique determinants that dictate coregulator interactions. This mechanism parallels the code embedded within the LxxLL/ΦxxΦΦ motifs that confers interaction specificity to coactivators and corepressors (Chang et al., 1999; Darimont et al., 1998; Hu and Lazar, 1999; McInerney et al., 1998; Nagy et al., 1999; Norris et al., 1999; Perissi et al., 1999; Sauvé et al., 2001).

We have shown that *in vitro*, the RORα LBD is in an active conformation favoring coactivator interaction and exerting an inhibitory influence on Hr binding.

This implies that the AF-2 helix masks the molecular determinants required for Hr binding, which may be otherwise unveiled in the presence of the corepressor under the appropriate conditions. For example, a tertiary protein may be necessary to anchor the AF-2 helix away from the surface of the LBD and allow Hr binding. Alternatively, phosphorylation may also be an important component influencing the dynamics of the AF-2 helix and enhancing Hr binding, thus shifting RORα into a repressed state. It has previously been shown that the affinity of peptides encoding LxxLL motifs for RORα is increased in the presence of Ca^{+2}/calmodulin-dependent protein kinase IV (CamKIV) (Kane and Means, 2000).

Convergence of ROR and Hr function *in vivo*. The functional significance of the interaction between Hr and RORα described in this study is clearly demonstrated by the degree to which Hr can repress RORα-mediated transcriptional activation and is likely to be of biological importance. Mutations in the gene encoding RORα result in the *staggerer* phenotype, which is characterized by severe ataxia and defects in both Purkinje and granule cells (Dussault et al., 1998; Hamilton et al., 1996; Matysiak-Scholze and Nehls, 1997; Steinmayr et al., 1998), suggesting that RORα is necessary for Purkinje cell survival. Interestingly, although *hr* is abundantly expressed in cerebellar granule cells, it is not present in Purkinje cells (Thompson, 1996). This predicts that in Purkinje cells where RORα activity is essential for survival, the receptor can function optimally. Given the developmental and tissue-specific expression of Hr (Ahmad et al., 1998; Thompson, 1996) and members of the ROR family (Giguère, 1999), Hr likely acts as a developmental and tissue-specific inhibitor of ROR family members in which the level of Hr expression regulates the

amount of ROR activity. More importantly, the expression of Hr is hormonally regulated (Thompson, 1996), providing a means to control RORα activity in response to exogenous stimuli. Notably, T_3 also influences cerebellar development, predicting the convergence of ROR and thyroid hormone signaling pathways during the development of the cerebellum (Hamilton et al., 1996). These results provide the first direct evidence linking T_3-dependent and ROR-dependent developmental processes.

Conclusion. The identification of Hr as a potent repressor of RORα transcriptional activity and the investigation into the molecular mechanisms regulating the interaction between the two proteins have revealed significant new insights into how RORα regulates gene expression. We have shown that RORα constitutive activity is likely dependent on the presence of an endogenous ligand and that a new class of nuclear receptor corepressors, represented here by Hr, can modulate that activity. More importantly, we have demonstrated that the interaction between Hr and nuclear receptors also requires specific determinants encoded within the AF-2 helix, a surprising finding in view of the results of previous studies attributing an inhibitory role to the AF-2 helix in nuclear receptor/coregulator interactions. Finally, the observation that Hr inhibits the transcriptional activity of a liganded receptor (RARα-R) suggests that this repression mechanism is likely to be shared by other members of the nuclear receptor family. The mechanism is also likely to be of physiological importance as transcriptional repression in the absence or presence of ligand constitutes an essential molecular pathway through which nuclear receptors control development and homeostasis (Hu and Lazar, 2000; Jepsen et al., 2000; Koide et al., 2001).

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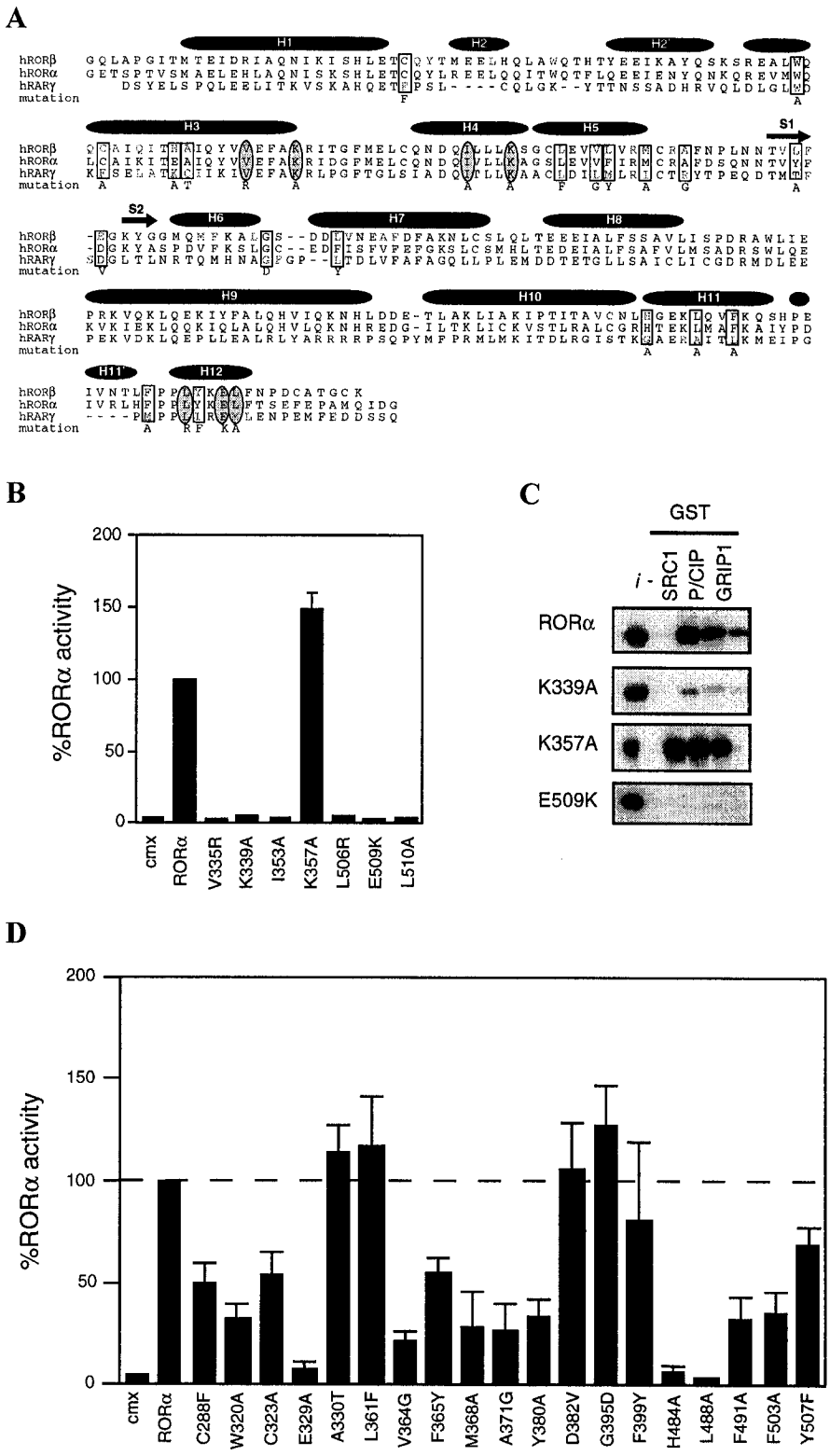
Figure 1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200
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RORα
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K339A
I353A
K357A
L506R
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L510A
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SRC1
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C323A
E329A
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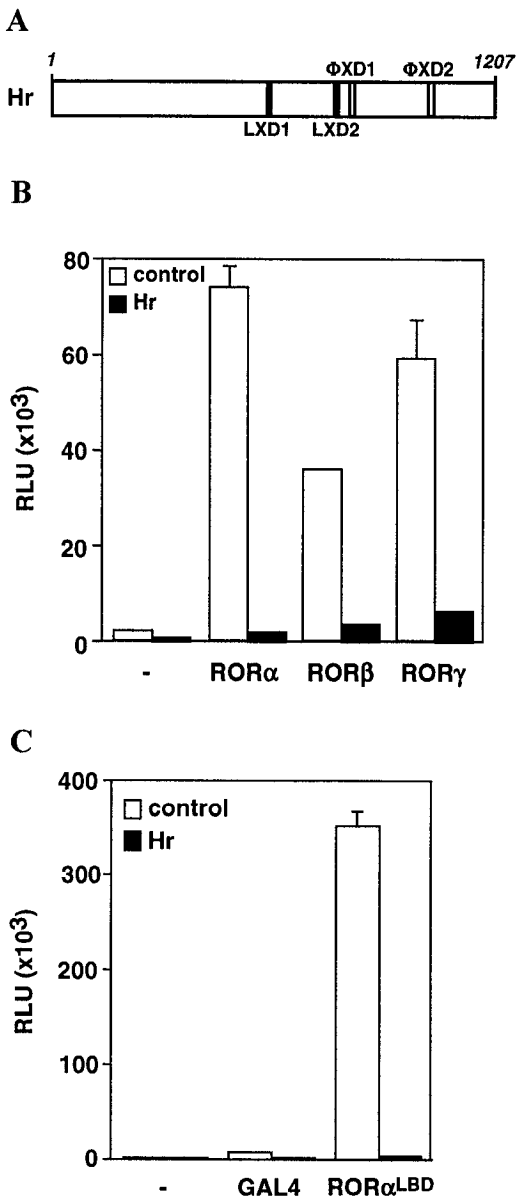
V364G
F365Y
M368A
A371G
Y380A
D382V
G395D
F399Y
H484A
L488A
F491A
F503A
Y507F

Figure 1. RORα shares common structural and functional determinants with classic nuclear receptors. (A) Primary sequence of RORβ, RORα and RARγ ligand binding domains. Amino acids involved in the LBP identified by crystallographic analysis are highlighted in red. Amino acids essential for AF-2 activity and known to participate in ligand binding targeted for site-directed mutagenesis are circled and boxed, respectively. The respective amino acid change is indicated below. The secondary structure is represented by black bars for the α-helices and arrows for the α-sheets. (B) RORαhydrophobic cleft mutants (V335R, K339A, I353A) and AF-2 helix mutants (L506R, E509K, L510A) are transcriptionally inactive in transfected COS-1 cells, with the exception of the cleft mutant K357A. Normalized values are calculated in terms of % RORa activity with respect to wild type. These results are the average of 3 independent experiments. (C) Binding of RORa and hydrophobic cleft (K339A, K357A) and AF-2 helix (E509K) mutants to SRC proteins. GST-SRC1a^{RID}, GST-p/CIP^{RID}, GST-GRIP1^{RID} fusion proteins were coupled to Sepharose beads incubated with ^{35}S-labeled RORa, RORα^{K339A}, RORα^{K357A}, RORα^{E509K}. The input lane (*i*) represents 10% of total lysate included in the binding reaction. (D) Cos-1 cells were cotransfected with RORα LBP mutants and REα2$_3$-TkLuc reporter. Normalized luciferase values are expressed in % activity with respect to wild type RORα. These results are the average of three independent experiments.

Figure 2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ΦXD2
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Hr
LXD1
LXD2
B
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Hr
RLU (x10³)
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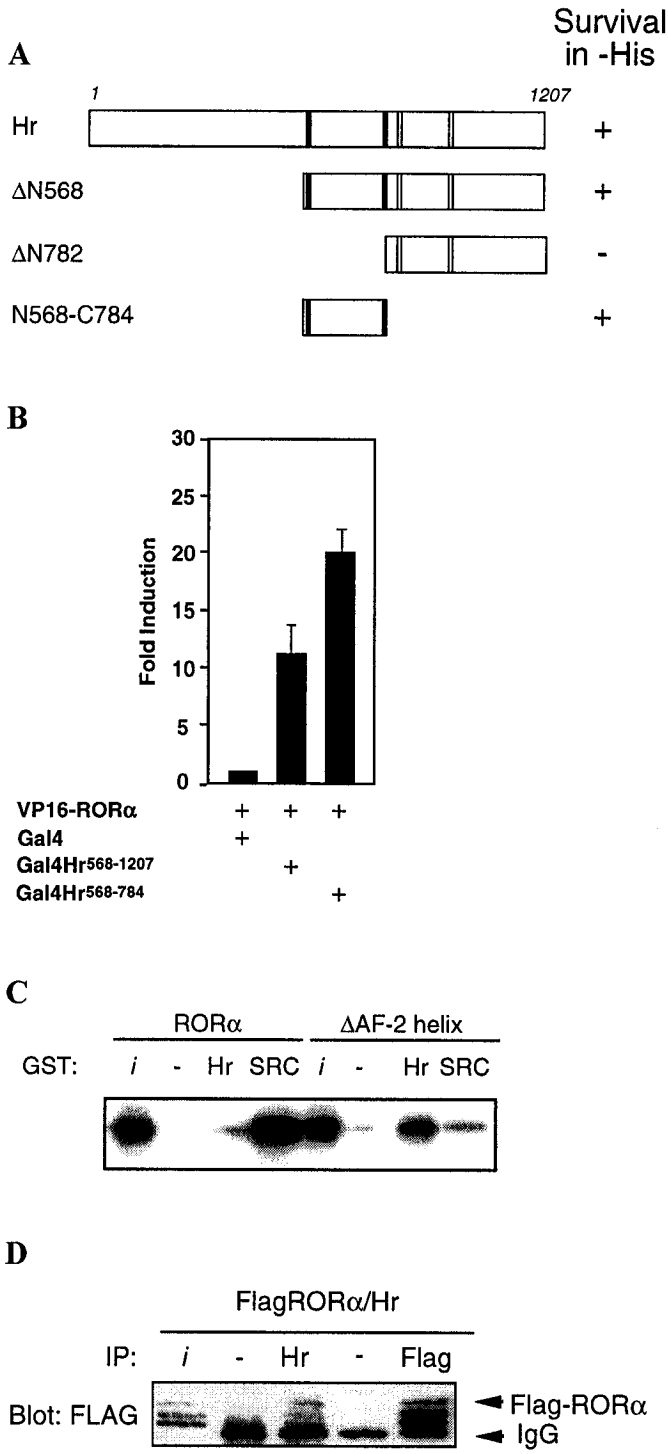
60
40
20
0
-
RORα
RORβ
RORγ
C
control
Hr
RLU (x10³)
400
300
200
100
0
-
GAL4
RORα^LBD

Figure 2. Hr represses ROR transcriptional activation. (A) Schematic representation of the Hr protein containing two LxxLL motifs (LXD1 and LXD2) and two ΦxxΦΦ motifs (ΦxD1 and ΦxD2). The numbers above indicate amino acid positions. (B) Hr represses RORα, β, γ constitutive transcriptional activities. Cos-1 cells were cotransfected with hRORα, mRORβ, mRORγ, and RORE$\alpha 2_3$-TKLUC in the absence (open bars) or the presence (black bars) of Hr. (C) Hr represses RORα activity on a heterologous promoter through its LBD. Schematic representation of the Gal4-RORαLBD, numbers above indicated the amino acid positions. Cos-1 cells were cotransfected with Gal4-RORαLBD, Hr and UAS_2-TkLuc. Normalized values are represented as relative luciferase units (RLU). A representative experiment of three independent experiments is shown. Error bars represent the standard deviation between duplicate samples.

Figure 3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Hr
+
ΔN568
+
ΔN782
-
N568-C784
+
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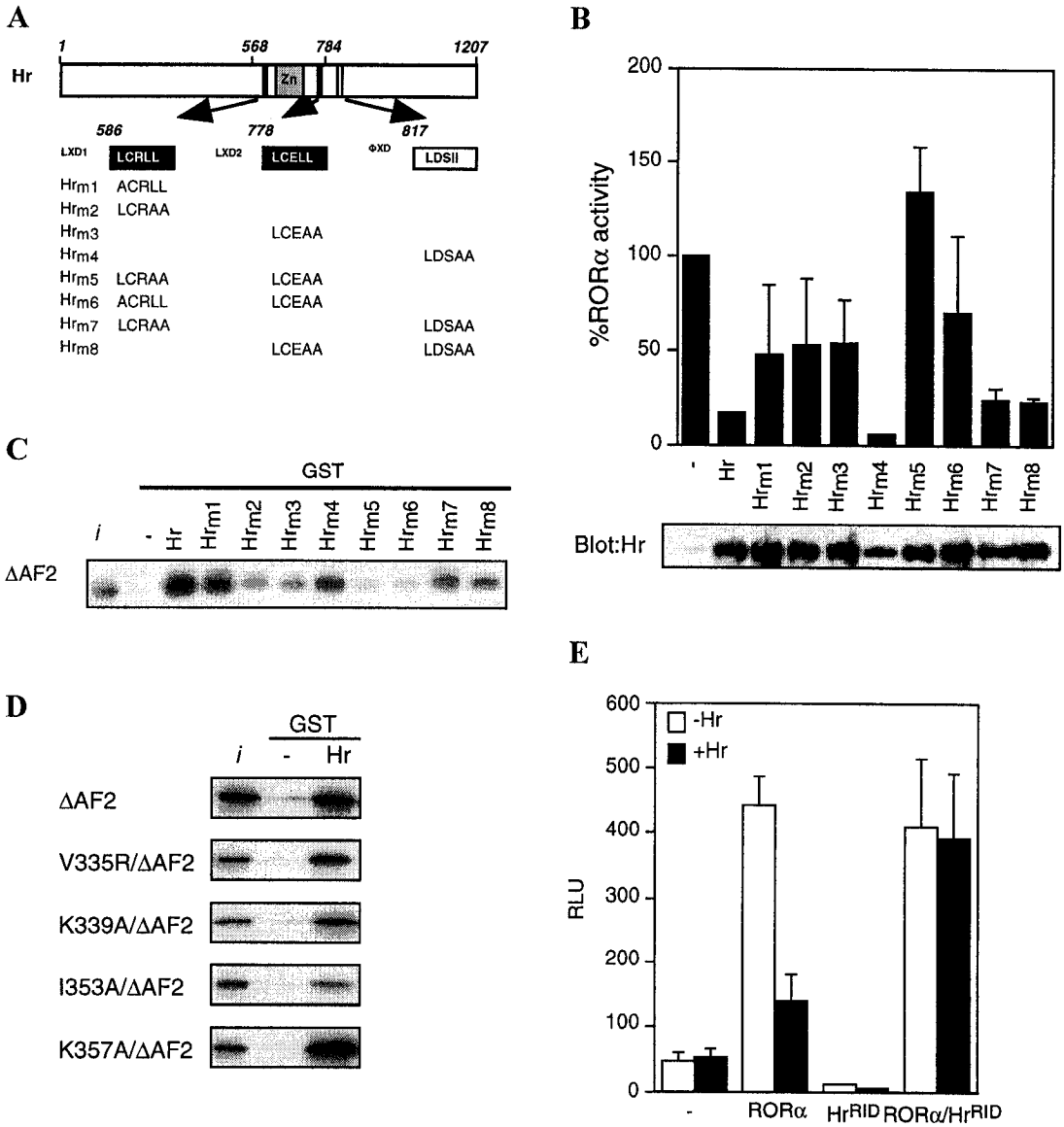
25
20
15
10
5
0
VP16-RORα + + +
Gal4 +
Gal4Hr568-1207 +
Gal4Hr568-784 +
C
RORα
ΔAF-2 helix
GST: i - Hr SRC i - Hr SRC
D
FlagRORα/Hr
IP: i - Hr - Flag
Blot: FLAG
Flag-RORα
IgG

Figure 3. Determinants involved in HR:RORα interaction. (A) A domain of Hr encoding two LxxLL motifs is sufficient for interaction with RORα. Results of yeast two hybrid assay with Hr deletion derivatives. The indicated Hr fragments were expressed as fusion proteins with the LexA DBD and tested for interaction with the RORa LBD fused with the VP16 activation domain. + indicates survival in the absence of histidine. (B) Cos-1 cells were cotransfected with Gal4-$Hr_{568-1207}$, Gal4-$Hr_{568-784}$, VP16-RORα, and UAS_2-TkLuc. Normalized values are expressed in fold induction. (C) The AF-2 helix inhibits Hr binding to RORα *in vitro*. *In vitro* translated and labeled RORα and RORαΔAF-2 were assayed for interaction with GST-$SRC1^{RID}$ or GST-$Hr_{568-784}$ coupled to Sepharose beads. The input lane (*i*) represents 10% of total lysate included in each binding reaction. (D) Hr interacts with RORa *in vivo*. Cos-1 cells were transiently transfected with pCMX-FlagRORα and pRk5-mycHr. Cell lysates were subjected to immunoprecipitation (IP) with Hr antibody, Flag antibody, rabbit or mouse IgG (as negative controls), followed by immunoblotting with anti-Flag. The input lane (i) represents 20% of lysate used in each IP.

Figure 4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ΦXD
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Hrm3 LCEAA
Hrm4 LDSAA
Hrm5 LCRAA LCEAA
Hrm6 ACRLL LCEAA
Hrm7 LCRAA LDSAA
Hrm8 LCEAA LDSAA
B
%RORα activity
200
150
100
50
0
-
Hr
Hrm1
Hrm2
Hrm3
Hrm4
Hrm5
Hrm6
Hrm7
Hrm8
Blot:Hr
C
GST
i
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Hr
Hrm1
Hrm2
Hrm3
Hrm4
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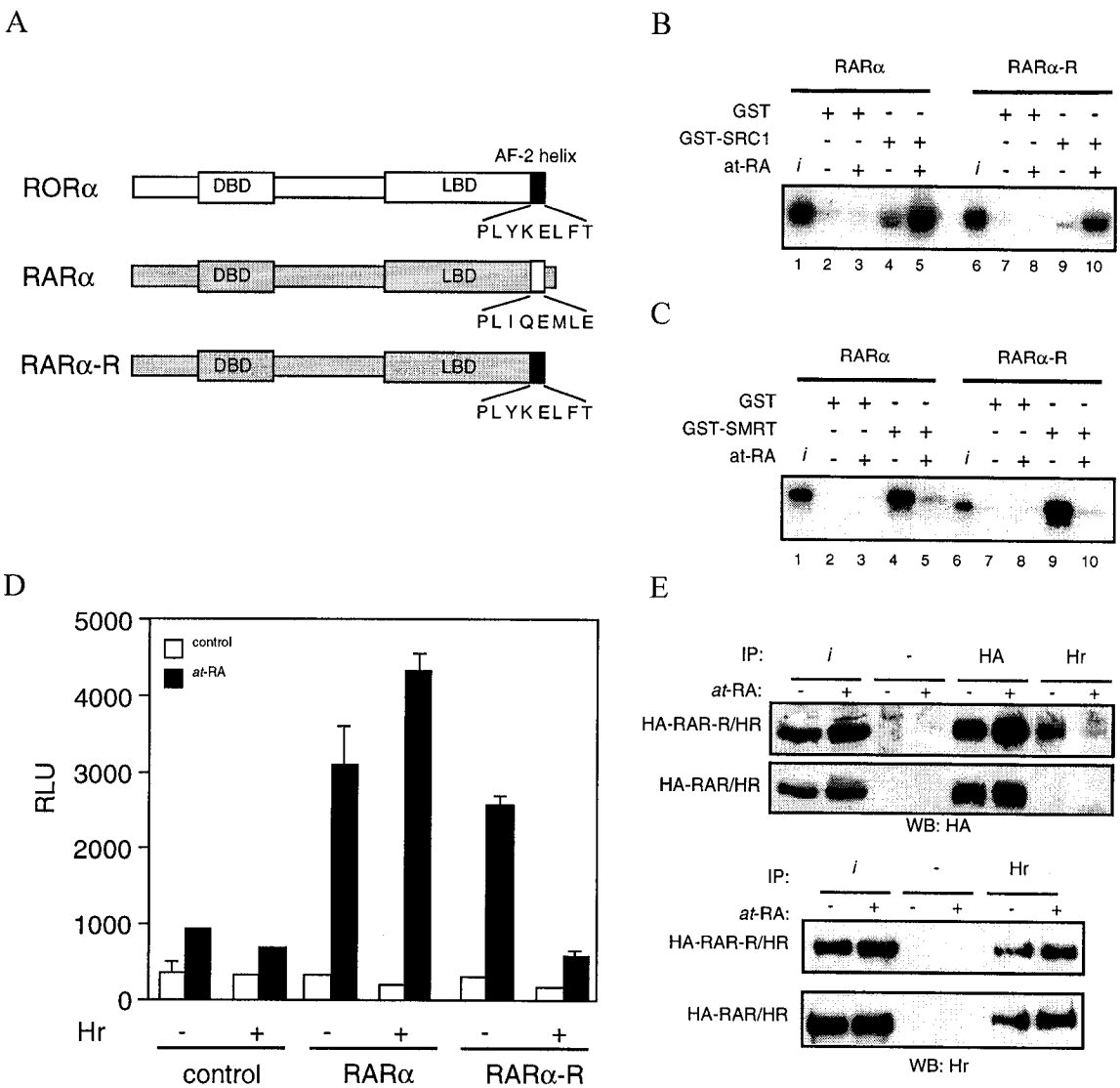
ΔAF2
V335R/ΔAF2
K339A/ΔAF2
I353A/ΔAF2
K357A/ΔAF2
E
-Hr
+Hr
RLU
600
500
400
300
200
100
0
-
RORα
HrRID
RORα/HrRID

Figure 4. Hr repression requires intact LxxLL motifs. (A) Schematic representation of the Hr protein. Hr_{m1}-Hr_{m6} encoding point mutations of the LXD1, LXD2 and FXD1 motifs are represented. (B) Top, Hr and Hr_{m1}-Hr_{m8} expression plasmids were cotransfected into Cos-1 cells with RORα and RORE$\alpha 2_3$-TkLuc reporter. Normalized values are expressed in %ROR activity. Results are the average of three independent experiments. Bottom, Cos-1 cells were transiently transfected with pRK5-mycHr wild type and mutant expression vectors. Extracts were immunoblotted with Hr antibody. (C) Hr repression correlates with ROR binding. GST-Hr and GST-Hr_{m1}-Hr_{m8} were coupled to Sepharose beads and incubated with ^{35}S-labeled RORαΔAF2 mutant, in a GST pull-down assay. The input lane (*i*) represents 10% of total lysate included in each binding reaction. (D) Hr interaction is not mediated through residues of the hydrophobic cleft. ^{35}S-labeled hydrophobic cleft mutants (V335R, K339A, I353A, K357A)/ΔAF2 were assayed for interaction with GST-Hr in a pull-down assay as above. (E) Hr^{RID} doesn't compete with endogenous coactivators. Cos-1 cells were transiently transfected with RORα, Hr and Hr^{RID} expression plasmids. Normalized values are expressed as relative luciferase units (RLU). Error bars represent the standard deviation between duplicate samples. This is one representative experiment of three.

Figure 5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AF-2 helix
RORα
DBD
LBD
PLYKELFT
RARα
DBD
LBD
PLIQEMLE
RARα-R
DBD
LBD
PLYKELFT
B
RARα
RARα-R
GST
GST-SRC1
at-RA
1 2 3 4 5 6 7 8 9 10
C
RARα
RARα-R
GST
GST-SMRT
at-RA
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D
control
at-RA
RLU
5000
4000
3000
2000
1000
0
Hr
control
RARα
RARα-R
E
IP:
at-RA:
HA-RAR-R/HR
HA-RAR/HR
WB: HA
IP:
at-RA:
HA-RAR-R/HR
HA-RAR/HR
WB: Hr

Figure 5. RORα AF-2 helix dictates specificity of Hr repression function. (A) Schematic representation of RORa and RARa, whose AF-2 helix is represented by a solid and an open box, respectively. RARα-R is a chimeric RARα encoding the RORa AF-2 helix. GST pull-down assays. ^{35}S-labeled RARα and RARα-R were incubated with GST, GST-SRC1^{RID} (B) or GST-SMRT^{RID} (C) in the absence (ethanol) or the presence of 10^{-6}M *all-trans* retinoic acid. Input represents 10% of the labeled protein used in a binding reaction. (D) Cos-1 cells were cotransfected with TREp$_3$-TkLuc, pCMX (control), hRARα/hRXRα (RARα), hRARα-R/hRXRα (RARα-R) in the absence (-) or the presence of Hr (+). Cells were treated with ethanol (open bars) or with 10^{-8} M *all-trans* retinoic acid (closed bars). Normalized values are expressed in relative luciferase units (RLU). Error bars represent the standard deviation between duplicate samples. This is a representative experiment of a total of three independent experiments. (E) Hr interacts with RAR-R. Cos-1 cells were transiently transfected with pRk5-mycHr, pCMX-HA-RARα-R or pCMX-HA-RARα. Cells were treated with ethanol (-) or 10^{-8} M *all-trans* retinoic acid (+). Cell lysates were subjected to immunoprecipitation (IP) with HA antibody, Hr antibody or rabbit IgG (as negative control), followed by immunoblotting with anti-HA or anti-Hr. The input lanes (*i*) represents 40% of lysate used in each IP.

Chapter IV. Ubiquitin-Proteosome Pathway Regulation of RORα Orphan Nuclear Receptor

Preface

In the previous two chapters, we discussed the molecular mechanisms governing RORα DNA binding, transcriptional activation as well as repression. In this chapter we describe another mechanism affecting RORα transcriptional activity, namely regulation of RORα protein stability by the ubiquitin-proteasome pathway. We demonstrate that the RORα is ubiquitin-conjugated and targeted for destruction by the ubiquitin-proteasome complex. Interestingly, blocking of proteasomal function with the peptide aldehyde MG-132, a pseudosubstrate of the 26S proteasome, led to inhibition of RORα transcriptional activity, suggesting that degradation and activation are intrinsically linked events. In addition, mutation of the ligand binding domain, thereby impairing ligand and coactivator binding, led to a loss of proteolytic degradation and an increase in protein stability, suggesting that ligand binding and subsequent binding of cofactor proteins may be involved in recruiting the ubiquitin-proteasome complex. The ubiquitin-proteasome complex plays an important role in regulating RORα protein stability, and concomitantly regulates nuclear receptor transcriptional activity by controlling turnover and promoter occupancy.

Abstract

Nuclear receptors are short-lived hormone-inducible transcription factors whose turnover is mediated by the ubiquitin-proteasome complex, in a ligand-dependent manner. It has been demonstrated that ubiquitin ligases are recruited to the activation complex, freeing the promoter of transcription factors and hence dampening gene expression. In addition, some coactivators exhibit dual action, functioning both as activators and ubiquitin-ligases. Proteolysis can be envisaged as a break on nuclear receptor mediated transcriptional activation, ensuring the appropriate hormonal response.

The RORα orphan nuclear receptor regulates the transcription of a myriad of genes involved in various cellular processes, such as cellular differentiation, including myogenesis and adipogenesis. In this study, we demonstrate that RORα undergoes ubiquitin-mediated proteasomal degradation. RORα degradation is blocked upon treatment of mammalian cells with the MG-132 proteasome inhibitor, resulting in the accumulation of transcriptionally inactive ubiquitin-conjugated receptor. We have previously demonstrated by mutational analysis, that RORα activity is dependent on the integrity of the ligand binding pocket and the coregulator-binding surface, and is likely regulated by an unidentified endogenous ligand. Mutation of these two critical domains renders the receptor transcriptionally inactive with strikingly increased protein stability, in comparison to wild type receptor. This data suggests that, similarly to classical hormone nuclear receptors, both endogenous ligand and

coregulator binding perpetuate RORα degradation. The ubiquitin-proteasome pathway not only regulates RORα protein stability but also controls its transcriptional response by limiting promoter occupancy by this potent activator.

Introduction

The ubiquitin-proteasome pathway is the major system employed by eukaryotes for the selective degradation of cellular proteins. The ubiquitin-proteasome pathway mediates degradation of a vast array of short-lived proteins, instrumental to the proper functioning of a number of cellular processes including cell cycle regulation, differentiation, development, signal transduction, transcription and chromosomal stabilization, in addition to degradation of abnormal or misfolded proteins (reviewed in Ciechanover, 1998; Glickman and Ciechanover, 2002; Kornitzer and Ciechanover, 2000; Voges et al., 1999). Proteolytic degradation by the ubiquitin-proteasome system involves ATP-dependent covalent attachment of a macromolecular chain of ubiquitin (ub) molecules to the target protein, followed by degradation through the multicatalytic 26S proteasome. The conjugation of ub, a highly conserved 8.6 kDa protein, to its target protein is mediated by the serial action of three enzymes: the E1 Ub-activating enzyme (UBA1) activates ubiquitin in an ATP-dependent manner; the E2 Ub-conjugating enzymes (UBCs) catalyze the attachment of Ub to the substrate protein; and the E3 Ub-ligases serve as a scaffold between E2 and the substrate, and provide recognition specificity of the substrate. Ubiquitinylation of a substrate is reversible and ubiquitin moieties can be cleaved from a target protein by deubiquitinating enzymes (DUB). DUBs assure that the cell is not depleted of an Ub pool. A protein tagged with a polyubiquitin chain is recognized and degraded by the 26S proteasome complex. This complex is composed of a 19S regulatory

subcomplex, consisting of a 'lid' subunit and a 'base' subunit, the latter containing 6 ATPases required for the degradation executed by the 20S catalytic subcomplex (Glickman and Ciechanover, 2002; Kornitzer and Ciechanover, 2000).

The ub-proteosome pathway plays a very important role in transcription and is emerging as a key regulator of eukaryotic mRNA synthesis, controlling the stability of both transcription factors, RNA polymerase II enzyme, and mRNA (reviewed in Conaway et al., 2002; Desterro et al., 2000; Laroia et al., 1999; Thomas and Tyers, 2000). Nuclear receptors are short-lived transcription factors whose turnover is mediated by the ub-proteasome complex (reviewed in Dennis et al., 2001). A number of nuclear receptors, including ER, PR, GR, RAR, RXR, T_3R and PPARγ are degraded in a ligand-dependent fashion (Boudjelal et al., 2000; Dace et al., 2000; Hauser et al., 2000; Lonard et al., 2000; Nomura et al., 1999; Syvala et al., 1996; Wallace and Cidlowski, 2001). Degradation of VDR and PXR reportedly occurs in a ligand-independent fashion, signaled by unstable interactions of steroid receptors with heat shock proteins (Li et al., 1999; Masuyama et al., 2002). In addition to ligand binding, phosphorylation of nuclear receptors by signal transduction pathways, and coregulator binding also serve as signals to the ub-proteasome complex, targeting the receptor for degradation. Corepressors and coactivators, such as NCoR corepressor, SRC (Steroid Receptor Coactivator) coactivator family members, and CBP (CREB-binding protein) are also substrates for proteasomal degradation (Baumann et al., 2001; Lonard et al., 2000; Zhang et al., 1998).

Ligand influences the stability of nuclear receptors by inducing a conformational change that permits cofactor docking. A number of these coregulators have been

identified as ub-proteasome or ubiquitin-like pathway enzymes, with a role in both proteasomal degradation as well as transcriptional activation. The E3 ub-ligases RSP5/RPF1 and E6-AP (E6-associated protein), the SUMO-conjugating enzyme UBC9, and the ATPase subunit of the 26S proteosome SUG1/TRIP1 (suppressor of Gal4/thyroid hormone receptor interacting protein-1) coactivate nuclear receptor transactivation, while simultaneously mediating their degradation (Gottlicher et al., 1996; Imhof and McDonnell, 1996; Lee et al., 1995; Nawaz et al., 1999; Rubin et al., 1997; von Baur et al., 1996). This dual action suggests that the ub-proteasome pathway plays a regulatory role in receptor-mediated transcription. Evidence that transcriptional activation and protein degradation occur concomitantly is further supported by the loss of nuclear receptor-mediated transcriptional activation observed upon inhibition of the 26S proteasome function (Lonard et al., 2000). This suggests that the ub-proteasomal complex is integral to nuclear receptor-mediated transcription. Downregulation of an activation complex may be required for the exchange of coactivator complexes leading to disruption of the pre-initiation complex, thereby allowing transcriptional elongation to proceed. The cell can then recycle components of the activation complex necessary for the initiation of a second round of transcription. This pathway provides a means of preventing the overstimulation by hormone.

The retinoic acid related orphan receptor (RORα) {NR1F1} is a member of the ROR subfamily, which also includes RORβ {NR1F2}, and RORγ {NR1F3}, each regulating diverse physiological processes. Genetic ablation of the *rora* gene leads to the *staggerer* phenotype, a recessive ataxic mouse described 40 years ago (Herrup

and Mullen, 1979; Sidman et al., 1962). The ataxic phenotype is caused by massive neurodegeneration of Purkinje cells in the cerebellum (Dussault et al., 1998; Hamilton et al., 1996; Steinmayr et al., 1998). RORα knock-out mice serve as a model for age-related degenerative pathologies, as these exhibit greater susceptibility to atherosclerosis, display immunodeficiencies, linked to an overexpression of inflammatory cytokines, abnormal formation and maintenance of bone, and changes in muscle differentiation (reviewed in Jarvis et al., 2002; Kopmels et al., 1992; Lau et al., 1999; Mamontova et al., 1998; Trenkner and Hoffmann, 1986). RORβ is predominantly found in neuronal cells and is involved in processing sensory information, as well as being implicated in the regulation of the circadian rhythm (André et al., 1998). The third member of this subfamily, RORγ has an important immunological role, regulating thymopoeisis and lymph node organogenesis (Kraichely et al., 2000).

RORα is a potent transcriptional activator, mediating the expression of target genes through a consensus AGGTCA half site motif, flanked by an 5'A/T rich sequence, termed an ROR response element (RORE) (Giguère et al., 1995). This RORE element also serves as a response element for Rev-ErbAα and RVR orphan nuclear receptors. Although RORα and Rev-ErbAα/RVR recognize overlapping gene networks, they oppositely regulate gene expression, the latter being a constitutive repressor. This was elegantly demonstrated by a study demonstrating that RORα and RVR control the expression, as well as the oncogenicity, of the N-myc proto-oncogene via a RORE element (Dussault and Giguère, 1997). RORα and RevErbAα/RVR also recognize a second type of response element comprising of a

direct repeat of the consensus half-site motif separated by two nucleotides (DR2). Rev-ErbAα/RVR preferentially forms homodimers on this element, whereas RORα lacks the essential dimerization determinants in its DNA-binding domain, that are required for homodimer formation (Moraitis and Giguère, 1999).

RORs activate transcription in the absence of exogenously added ligand. Recently, resolution of the crystal structures of RORα and RORβ ligand binding domains (LBDs), in combination with mutagenesis assays of the RORα ligand binding domain, suggest that members of the ROR family are regulated by ligand (Kallen et al., 2002; Moraitis et al., 2002; Stehlin et al., 2001). Regulation of RORα transcriptional activity is also mediated through coregulator recruitment. RORα has been shown to interact with members of the SRC family, and the p300/ CBP co-integrators, where SRC-2/GRIP1 potentiates RORα-mediated transcriptional activity (Atkins et al., 1999; Lau et al., 1999). Repression of RORα activity is not only regulated by a passive mechanism, through competition with RevErbAα/RVR, but also by an active mechanism. RORα, like many members of the nuclear receptor superfamily, interacts with NCoR and SMRT corepressors. Interestingly, RORα transcriptional activity is also strongly repressed by the AF-2 specific Hairless corepressor (Moraitis et al., 2002). Signal transduction pathways, triggered by environmental factors and events at the cell membrane, provide an additional level of nuclear receptor regulation. Although RORα has not been shown to be directly phosphorylated, its activity is potentiated by Ca+2/calmodulin kinase IV (CamKIV), in response to calcium (Kane and Means, 2000).

As the mechanisms regulating RORα activity begin to unravel, we demonstrate in this study that the ub-proteasome pathway provides an additional layer of regulation of RORα-mediated transcriptional activity. We observed that RORα protein expression level increases upon inhibition of the 26S proteosome complex with the MG-132 peptide aldehyde. RORα degradation occurs following ubiquitin conjugation, likely signaled by an endogenous ligand and recruitment of cofactors to the activated receptor. Proteasomal inhibition is detrimental to RORα transcriptional activity, suggesting that the ubiquitin-proteasome pathway is an integral part of RORα-mediated transcription.

Material and Methods

Plasmids. pCMX-hRORα1 wild type and ligand binding domain mutants L361F, V364G, K357A, E509K as well as pCMX-Flag-hRORα1 have been previously described (Moraitis et al., 2002). Amino terminal deletion mutants of hRORα1, RΔN12, RΔN25 and RΔN35 have been described elsewhere (Giguère et al., 1995). pCMXGAL4-RORαΔNTD/ΔHinge (described elsewhere as Gal4-RORαLBD (Moraitis et al., 2002)) encoding amino acids 270-523 was constructed by cloning in frame an *Eco*RV/*Bam*HI fragment from pCMXhRORα1 downstream of the Gal4 DBD sequence. pCMV-HA-Ubiquitin, consists of a octameric ubiquitin construct, each ubiquitin is preceded by at its N-terminus by an HA tag, described in (Treier et al., 1994).

Cell Culture and Transient Transfection. Cos-1 cells obtained from the American Type Culture Collection were cultured in Dulbecco's Minimal Essential Medium (DMEM) containing penicillin (25 U/ml), streptomycin (25 U/ml) and 10% fetal calf serum at 37°C with 5% CO_2. Twenty-four hours prior to transfection the cells were split and seeded in 12 well-plates. The cells were transfected with FuGENE 6 Transfection Reagent (Roche Diagnostics), following the protocol supplied by the manufacturer. A total of 1 μg of DNA was transfected per well including 0.1 μg of pCMX-hRORα1, 0.5 μg of reporter plasmid, and 0.25 μg of internal control pCMVβGal. Cells were treated with ethanol (vehicle) or 0.1, 0.5, 1.0 μM MG-132 for 16-24 hours, as specified in figure legends. Cells were harvested and assayed for luciferase and β-galactosidase. Normalized values are expressed in terms

of relative luciferase units (RLU). Error bars represent standard deviations between duplicate samples. Each graph is one representative experiment of a total of three independent experiments.

Co-Immunoprecipitation and Immunoblotting Assays. Cos-1 cells in 10 cm dishes were transiently transfected as described above, with 10 μg of Flag-RORα and HA-Ub, and treated with ethanol (vehicle) or 1 μM MG-132 for 24 hours. Cells were lysed in IP buffer (1% NP-40, 10 % glycerol, 150 mM NaCl, 50 mM Tris-HCl pH 7.5) supplemented with protease inhibitor cocktail (Complete Mini EDTA-free, Roche Diagnostics). Lysates (containing a total of 250 μg of protein) were incubated with 5 μg Flag antibody (Sigma) overnight at 4°C, with gentle rotation. Proteins were collected on protein G-Sepharose for 2hrs at 4°C with mild rotation and then washed three times with ice-cold low salt buffer (1% NP-40, 50 mM Tris-HCl, pH8.0). Immunoprecipitates were resolved by SDS-PAGE, transferred to a Hydrophobic polyvinylidene difluoride (PVDF) membrane (Amhersham Pharmacia Biotech) and immunoblotted with Flag antibody or HA antibody (HA.11, Berkeley Antibody Company). Proteins were visualized with the POD chemiluminescence kit following manufacturer's instructions (Roche Diagnostics). Immunoblotting for detection of RORα and mutants, or actin, was similarly done using anti-RORα antibody (C-16) (Santa Cruz Biotechnology) and anti-actin antibody (I-19) (Santa Cruz), respectively. The lysates were prepared from transiently transfected Cos-1 harvested in modified RIPA buffer (50 mM Tris-HCl, pH7.4, 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1

mM Na_3VO_4, 1 mM NaF), resolved by SDS-PAGE, transferred and immunoblotted as described above.

***In vitro* degradation assay.** Cell extract was prepared from Cos-1 cells harvested in modified RIPA buffer. 5 μl of *in vitro* translated ^{35}S-methionine labeled RORα, using T_NT rabbit reticulocyte lysate (Promega, Madison, WI), was incubated with 50 μg cell extract, ethanol (vehicle), 50 μM MG-132, 20 μM lactacystin, 50 μg/ml EST, or 2 mM PMSF in a final volume of 50 μl degradation buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.2 mM DTT) for 2 hours at 37°C. Samples were resolved by SDS-PAGE. Gels were fixed and treated with fluorographic reagent Amplify (Amhersham Pharmacia Biotech), dried and exposed.

Results

The ubiquitin-proteasome complex degrades RORα. The use of pharmacological proteasomal inhibitors is an extremely useful tool in studying the ub-proteasomal degradation of short-lived proteins. Peptide aldehydes (MG-132) or natural products (lactacystin) act as pseudosubstrates that become covalently linked to the 26S proteosome and inactivate its chymotryptic and tryptic-like activities (Lee and Goldberg, 1998). We investigated the expression level of RORα in transiently transfected Cos-1 cells treated with the proteasomal inhibitor, MG-132. As shown in Fig. 1A, RORα is not endogenously expressed in Cos-1 cells, although overexpressed RORα is detected by immunoblotting with anti-RORα antibody. Blocking of the 26S proteosome with MG-132 leads to a markedly increased RORα protein expression level, suggesting that it may be a substrate of the ub-proteasome complex. We next used Cos-1 extract as a source of ub-proteasome complex in an *in vitro* degradation assay to determine whether *in vitro* translated and labeled RORα is proteolytically degraded. Downregulation of labeled RORα was observed upon incubation with Cos-1 extracts, and was subsequently blocked upon treatment with MG-132 inhibitor, suggesting that RORα is degraded by the 26S proteasome (Fig. 1B). In addition to MG-132, lactacystin an irreversible specific inhibitor of the 20S proteasome also blocked RORα degradation, as demonstrated by a marked increase in protein expression in comparison to vehicle treated sample (Fig. 1C). In contrast, the lysosomal specific cysteine protease inhibitor EST, as well as the nonspecific serine

protease inhibitor PMSF failed to stabilize RORα protein expression therefore suggesting that RORα is degraded by the 26S proteasome.

RORα proteasomal degradation is an integral part of transactivation potential. Substrates destined for proteasomal degradation are tagged by covalent attachment of a macromolecular ubiquitin chain. Co-immunoprecipitation of RORα and ubiquitin resulted in the appearance of high molecular weight ub-conjugated RORα complexes, in cells treated with MG-132 inhibitor (Fig. 2A). Given the absence of Ub-RORα complexes in untreated cells, Ub tagged RORα is likely rapidly degraded by the 26S proteosome, under normal conditions (Fig. 2A). Ubiquitin-mediated degradation of nuclear receptors and other transcription factors is tightly coupled to their transactivation potential and the potency of their activation domains, providing the cell with a 'suicide' mechanism to protect against deleterious levels of transcription. The ub-proteasome pathway plays an integral part in nuclear receptor-mediated transcription (Dennis et al., 2001). It has been demonstrated that this pathway is imperative for a functional hormone-mediated transcriptional response of the estrogen receptor (Lonard et al., 2000). We therefore assessed whether this finding could be extended to the potent transcriptional activity exhibited by RORα. As shown in Fig. 2, transient transfection of Cos-1 cells with RORα and a RORE-driven reporter results in a progressive inhibition of RORα-mediated transactivation in response to increasing concentrations of MG-132 inhibitor (Fig. 2B). Blocking of the 26S proteosome therefore leads to the accumulation of transcriptionally inactive ubiquitin-conjugated RORα. This suggests that a functional ub-proteasome pathway required for receptor turnover is critical for efficient transcriptional activation.

Putative endogenous RORα ligand signals the ubiquitin-proteasome pathway. It has been suggested that ligand is a signal to the ubiquitin-proteasome complex regulation of nuclear receptor degradation, such as ER, PR, RAR, TR, and RXR (Boudjelal et al., 2002; Boudjelal et al., 2000; Dace et al., 2000; Lonard et al., 2000; Nomura et al., 1999; Prufer et al., 2002; Syvala et al., 1996). A caveat in studying orphan receptors is the absence of a *bone fide* ligand. We have previously shown that mutation of key residues in the ligand binding pocket (LBP) led to a loss of transactivation potential, suggesting that binding of an endogenous ligand that would mediate this activity is hindered (Moraitis et al., 2002). In light of the recent resolution of the RORα LBD crystallographic data it was found that cholesterol was trapped in the ligand binding pocket, suggesting that it may be the physiological RORα ligand, thus confirming our model of a ligand-dependent RORα receptor (Kallen et al., 2002). It was demonstrated that cholesterol can potentiate the transcavtivation potential of RORα in transiently transfected cells, although whether its effect is direct remains to be determined. We had therefore used RORα^{V364G} and RORα^{L361F} mutants to simulate inactive and active receptor conditions, respectively given that we were unable to demonstrate any effect of cholesterol on RORα in our system. Equivalent residues of RORα^{V364G} in RORβ and RARγ have been shown to be involved in ligand binding, due to their close proximity to their cognate ligand, and affect the shape of the LBP of RORβ (Renaud et al., 1995; Stehlin et al., 2001). Transiently transfected Cos-1 cells were treated with proteasome inhibitor MG-132 and transactivation as well as protein expression levels of both wild type and mutant RORα were assayed (Fig. 3). RORα^{V364G} mutant is likely involved in ligand binding,

given its lack of transcriptional activity (Fig. 3A). In contrast, RORα^{L361F} is functional and activates transcription from a reporter gene, and is probably not required for ligand binding. Interestingly, RORα^{V364G} expression is greater than that of wild type in the absence of proteasomal inhibitor, demonstrating the transcriptional inactivity is independent of protein expression levels of this mutant. RORα^{L361F} expression is similar to that of wild type, given that this mutation likely does not affect the function of the receptor (Fig. 3B). Treatment of cells with MG-132 inhibitor increases the expression level of both wild type RORα and RORα^{L361F}, whereas no significant effect can be observed on RORα^{V364G} expression (Fig. 3B). This data suggests that transcriptionally inactive RORα^{V364G} is not degraded by the ub-proteasome complex, and suggests that degradation may be triggered by ligand binding.

Ligand binding induces a conformational change of the LBD resulting in an exchange of corepressor for coactivator complexes. Residues of the hydrophobic cleft at the surface of the ligand binding domain, together with residues of the AF-2 helix, form a coactivator binding surface (Feng et al., 1998). We and others have demonstrated that RORα transcriptional activity is dependent on the integrity of a functional coactivator binding surface (Atkins et al., 1999; Harris et al., 2002; Moraitis et al., 2002). To assess the importance of this interface in proteasomal-mediated RORα degradation, we tested the expression levels of an AF-2 deficient mutant (RORα^{E509K}), as well as a functional hydrophobic cleft mutant (RORα^{K357A}) as a positive control. As shown in Fig. 3A, the RORα^{K357A} mutant exhibits constitutive transcriptional activity, whereas RORα^{E509K} is transcriptionally inactive. We have previously shown that their transactivation potentials can be correlated to their ability

to interact with SRC coactivators (Moraitis et al., 2002). In a manner analogous to wild type RORα, RORα^{K357A} transcriptional activity is inhibited by MG-132, and in parallel the protein expression level increases (Fig. 3B), suggesting that this mutant is also degraded in a 26S proteasome dependent fashion. The transcriptionally inactive AF-2 deficient mutant RORα^{E509K} exhibits a stronger protein expression level than wild type RORα, which is not significantly affected by treatment with the MG-132 proteasomal inhibitor, suggesting that unlike its transcriptionally active counterparts, RORα^{E509K} is not rapidly degraded. The proteasomal-mediated degradation of RORα therefore requires an intact AF-2 domain, which may be involved in the recruitment of cofactor proteins that form part of the ub-proteasome complex. Mutation of this domain may inhibit binding of proteins, such as SUG1/TRIP1, a component of the 19S proteasome subcomplex which also functions as a nuclear receptor coactivator, and has been shown to interact with the RORα LBD (Atkins et al., 1999).

Proteins targeted for degradation by the ub-proteasome complex often contain a short hydrophilic stretch of at least 12 amino acids rich in proline (P), glutamic acid (E) or aspartic acid (D), serine (S) and threonine (T) residues, flanked by lysine, histidine, or arginine residues, termed a PEST motif. A PEST region serves as a proteolytic signal leading to rapid destruction of a given protein. Using a *PESTfind* program (*at.embnet.org/embnet/tools/bio/PESTfind/about.htm*), we identified three putative PEST sequences in RORα (Fig. 4A) (Rechsteiner and Rogers, 1996; Rogers et al., 1986). This algorithmic program scores the hydrophilicity of regions enriched in P, E/D, S, or T amino acids, disallowing positively charged residues, in a range of –50 to +50. A potential PEST motif is one that scores above +0, although one that

scores above +5.0 is considered a more probable candidate. There is a potential PEST motif scoring +6.88 encoded in the NTD of RORα, and three weak motifs encoded in the hinge domain with scores of –2.47, -6.89, and +0.68. To determine the involvement of the N-terminal PEST sequence in signaling RORα degradation, we generated three N-terminal deletion mutants, RORαΔ12, RORαΔ25 and RORαΔ35 (Fig. 4B). We also used Gal4-RORα fusion proteins, in which the PEST sequences of either the hinge domain were deleted alone (Gal4ΔHinge) or in combination with the N-terminal PEST motif (Gal4ΔNTD/Hinge) (Fig. 4D). These constructs were transiently transfected in Cos-1 cells, and their transactivation potential was assessed on a RORE- or UAS-driven reporter. All deletion proteins displayed potent transcriptional activity, which was inhibited by treatment of the cells with the MG-132 (Fig. 4B,D). Deletion of the N-terminal PEST motif does not affect RORα degradation, given that protein expression of RΔ25 is potentiated upon treatment with MG-132 inhibitor (Fig. 4C). This suggests that removal of the putative PEST sequences does not hinder the proteolytic signal that targets RORα for destruction by the 26S proteasome. We have demonstrated that recruitment of the ub-proteasome complex is dependent on an intact LBD and is not signaled by a PEST motif, rather the proteolytic signal may be emanating from a ligand-dependent cofactor. Proteasomal degradation of RORα is therefore closely linked to its activation state, and may provide a mechanism regulating RORα-mediated transcription.

Discussion

The ub-proteasome system is involved in many cellular processes including cell cycle regulation, signal transduction pathways, cellular differentiation, development, chromosomal silencing and regulation of transcription (Kornitzer and Ciechanover, 2000). This pathway regulates the turnover of many transcription factors, including members of the nuclear receptor superfamily (Dennis et al., 2001). Transcription factor activation and destruction are closely linked, providing the cell with an efficient suicide mechanism for attenuating transcription (Conaway et al., 2002). The more potently a given transcription factor activates transcription, the more rapidly it is ubiquitin-tagged and degraded. An inverse correlation has been established between the strength of an activation domain and the protein half-life (Molinari et al., 1999; Salghetti et al., 2001; Salghetti et al., 2000). Given that the RORα orphan nuclear receptor is a strong transcriptional activator, we investigated whether its potent activation domain is involved in the downregulation of the receptor by signaling to the ub-proteasome complex. In this study, we show that RORα is ubiquitin-conjugated and degraded by the ub-proteasome pathway. Treatment of cells with MG-132, a pseudosubstrate of the 26S proteasome that inhibits its catalytic function, results in a marked increase of RORα protein levels both *in vivo* and *in vitro*. Interestingly, blocking the ub-proteasome pathway also impairs RORα transcriptional activity, suggesting that degradation is an integral part of RORα-mediated transcription.

To date, nuclear receptor degradation by the ub-proteasome complex has been limited to studies of receptors that are regulated by a known ligand (Dace et al., 2000;

Li et al., 1999; Lonard et al., 2000; Nomura et al., 1999; Wallace and Cidlowski, 2001; Zhu et al., 1999). The ligand plays a key role in mediating substrate recognition by inducing the transconformation of the ligand binding domain allowing docking of proteins involved in the ub-proteasome pathway. The caveat in our study is the absence of a *bone fide* RORα ligand (Kallen et al., 2002). Despite the recent crystallographic data demonstrating that cholesterol occupies the ligand binding pocket of RORα, to date our attempts at demonstrating that cholesterol potentiates RORα activity have been successful. Mutagenesis assays have therefore been instrumental to the understanding of the mechanisms involved in RORα transcriptional activity. Mutations in the ligand binding pocket render the receptor transcriptionally inactive, suggesting that RORα is regulated by an endogenous ligand (Moraitis et al., 2002). The ligand binding pocket mutant RORα^{V364G} provides a means of hindering RORα transcriptional activity, and mimicking unbound receptor conditions. Strikingly, RORα^{V364G} exhibits greater protein expression than wild type RORα, and is unaffected by inhibition of the 26S proteosome complex, suggesting that proteolytic degradation requires an intact ligand binding domain. The AF-2 deficient mutant, RORα^{E509K}, is highly expressed irrespective of treatment with the proteosome inhibitor MG-132, suggesting that only a transcriptionally active receptor undergoes proteasomal degradation. Moreover, this also demonstrated that an intact coactivator binding surface is required for proteolytic degradation. Given that both activation and degradation are regulated by ligand, binding of a putative RORα ligand may not only recruit coactivator proteins necessary for transcriptional activation but may also recruit proteins of the ub-proteasome complex. This is the first example of

an orphan nuclear receptor that is degraded by the ub-proteasome pathway. This data suggests that this mechanism could be extended to all members of the nuclear receptor superfamily.

Recently, a number of ub-proteasome and ubiquitin-like pathway enzymes have been shown to be nuclear receptor coactivators. The E3 protein ligases, E6-AP, RPF1/RSP5, and UBC9 play a dual role as nuclear receptor coactivators and may be an integral component of the RNA polymerase II machinery. (Gottlicher et al., 1996; Imhof and McDonnell, 1996; Nawaz et al., 1999). RNA polymerase II recruits E3 ubiquitin ligases through phosphorylation of its C-terminal domain. In addition, the ATPase enzymes of the 19S proteosome subcomplex, namely SUG1 and SUG2, have recently been shown to associate with actively transcribed genes (Gonzalez et al., 2002; Ottosen et al., 2002). SUG1/TRIP1 binds directly to the activation domains of Gal4 and other transcription factors, and functions as a nuclear receptor coactivator (Chang et al., 2001; Lee et al., 1995; Melcher and Johnston, 1995; Rubin et al., 1997; von Baur et al., 1996). RORα has been shown to recruit SUG1/TRIP1 to its LBD in a yeast two-hybrid assay, although it is not yet known whether this putative coactivator can potentiate RORα transcriptional activity, or recruit the ubiquitin-proteasome complex for RORα degradation (Atkins et al., 1999). The involvement of SUG1 in the regulation of RORα transcriptional activity and stability requires further investigation.

Recognition of target proteins by the ub-proteasome complex is mediated through specific motifs which signals to E3 ubiquitin ligase enzymes that a given protein is to be tagged with a polyubiquitin chain. Many rapidly degraded regulatory proteins

contain PEST motifs, regions rich in proline, glutamic acid, serine and threonine residues. The GR nuclear receptor encodes a PEST motif, essential for ligand-mediated degradation, since point mutation of this motif abrogates down-regulation (Wallace and Cidlowski, 2001). RORα encodes a putative PEST sequence in its N-terminal domain and three weak sequences in the hinge region. We have demonstrated that these motifs are not required for proteasomal degradation of this orphan. RXR encodes PEST motifs in the N-terminal and hinge domains, similar to RORα, and mutation of these motifs does not affect proteolytic degradation of the receptor (Boudjelal et al., 2000). The absence of a functional PEST motif is not uncommon, given that a number of receptors, including ER and TR, are downregulated by the ub-proteasome complex despite the lack of this consensus signaling motif (Dace et al., 2000; Lonard et al., 2000). Proteolysis may also be triggered by phosphorylation and/or recruitment of cofactors. For example, ligand-dependent degradation of RARγ is dependent on its phosphorylation and dimerization states (Kopf et al., 2000). Ligand-dependent GR degradation is also signaled by phosphorylation, since a phosphorylation deficient mutant does not undergo proteolysis (Wallace and Cidlowski, 2001). Despite a number of putative consensus phosphorylation sites in RORα, the phosphorylation state of the receptor remains elusive. The specific signals that engage nuclear receptors into the ub-proteasome pathway have not yet been clearly delineated.

Transcription and degradation are closely linked events, where the cell sacrifices energy expenditure required for nuclear receptor degradation in exchange for a fail-safe mechanism against harmful levels of transcription (Conaway et al., 2002;

Thomas and Tyers, 2000). This interdependency of nuclear receptor-mediated transcription and ub-proteasome degradation has also been demonstrated by the requirement of ligand-dependent ERα transcription on the presence of the E1 ub-activating enzyme in the cell (Lonard et al., 2000). In addition to RORα, blocking proteasomal degradation impairs ER, TR and PR-mediated transcription, suggesting that this is a general mechanism regulating nuclear receptor function. To date, RORα transcriptional activity is known to be attenuated by passive repression, through competition for overlapping binding sites with the constitutive repressor Rev-ErbAα/RVR, and also by active repression mediated by the Hairless corepressor (Dussault and Giguère, 1997; Moraitis et al., 2002). In this study, we demonstrated that the ub-proteasome pathway provides another mechanism of regulating RORα transcriptional activity. RORα degradation is a means of protecting the cell against abnormal and deleterious levels of RORα-mediated transcriptional activation, by attenuating the expression of this potent activator.

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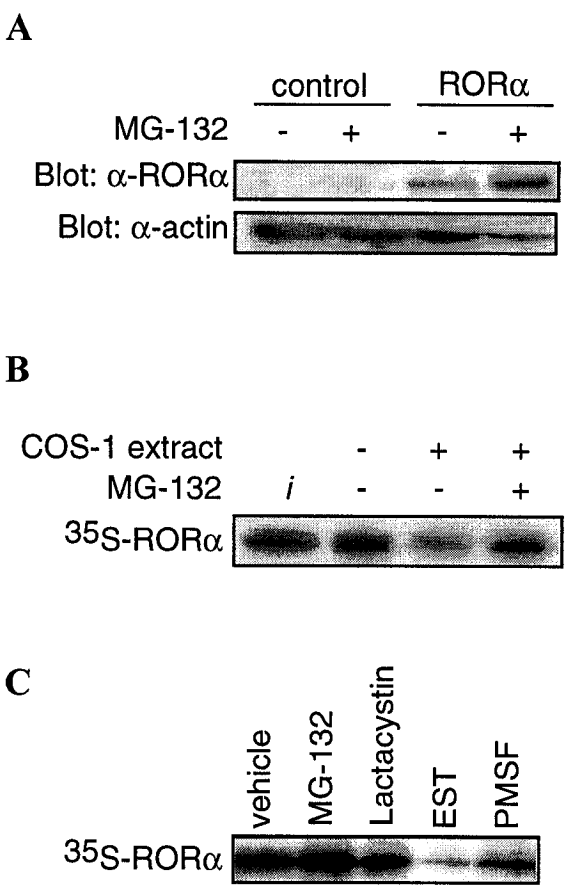
Figure 1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RORα
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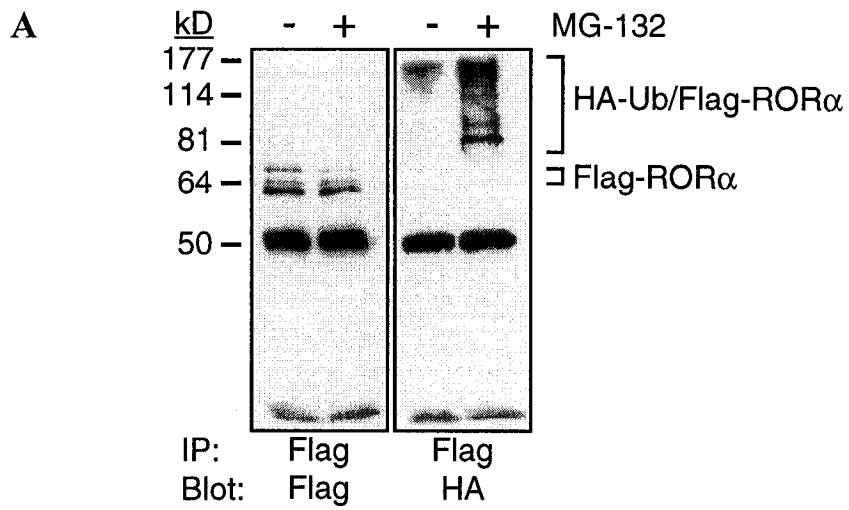
C
vehicle
MG-132
Lactacystin
EST
PMSF
35S-RORα

Figure 1. The ubiquitin-proteasome complex degrades RORα. (A) Cos-1 cells were transiently transfected with empty vector (control) or RORα expression plasmid, treated with ethanol (vehicle) or 1 μM MG-132 proteasome inhibitor. Cell lysates were resolved by SDS-PAGE and immunoblotted with anti-RORα or anti-actin antibody. (B) *In vitro* degradation assay of *in vitro* translated and labeled RORα, incubated with Cos-1 cell extracts, treated with ethanol (-) or 50 μM MG-132 inhibitor (+). The input (*i*) represents labeled RORα not subjected to the 37°C incubation required for the degradation reaction. (C) *In vitro* degradation assay of in vitro translated and labeled RORα incubated with Cos-1 extract in presence of vehicle (ethanol) or MG-132, lactacystin, EST or PMSF inhibitors as specified in Material and Methods.

Figure 2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Flag-RORα
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125
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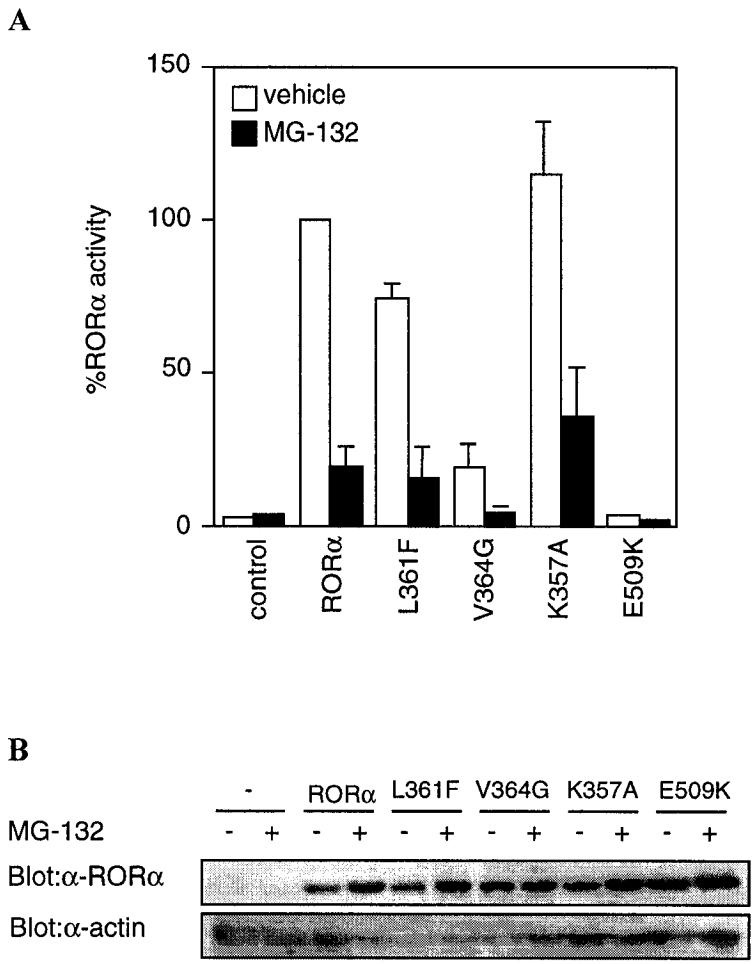
750
50
25
0
RLU (x10³)
vehicle
0.1 μM MG-132
0.5 μM MG-132
1.0 μM MG-132
control
RORα

Figure 2. The ubiquitin-proteasome pathway is involved in RORα transcriptional activity. (A) Cos-1 cells transiently transfected with HA tagged ubiquitin (Ha-Ub) and Flag tagged RORα, treated with ethanol (-) or 1 μM MG-132 proteasomal inhibitor (+). Lysates were subjected to immunoprecipitation with anti-Flag antibody and immunoblotted with anti-Flag or anti-HA antibodies. (B) Cos-1 cells were transiently transfected with empty vector (control) and RORα expression plasmid and transcriptional activity on a RORE$\alpha 2_3$-TkLuc reporter was assayed. Normalized values are expressed in relative luciferase units (RLU). Cells were treated with ethanol (vehicle) or increasing concentrations of MG-132 proteasome inhibitor. Error bars represent the standard deviation between duplicate samples. This is one representative experiment of three.

Figure 3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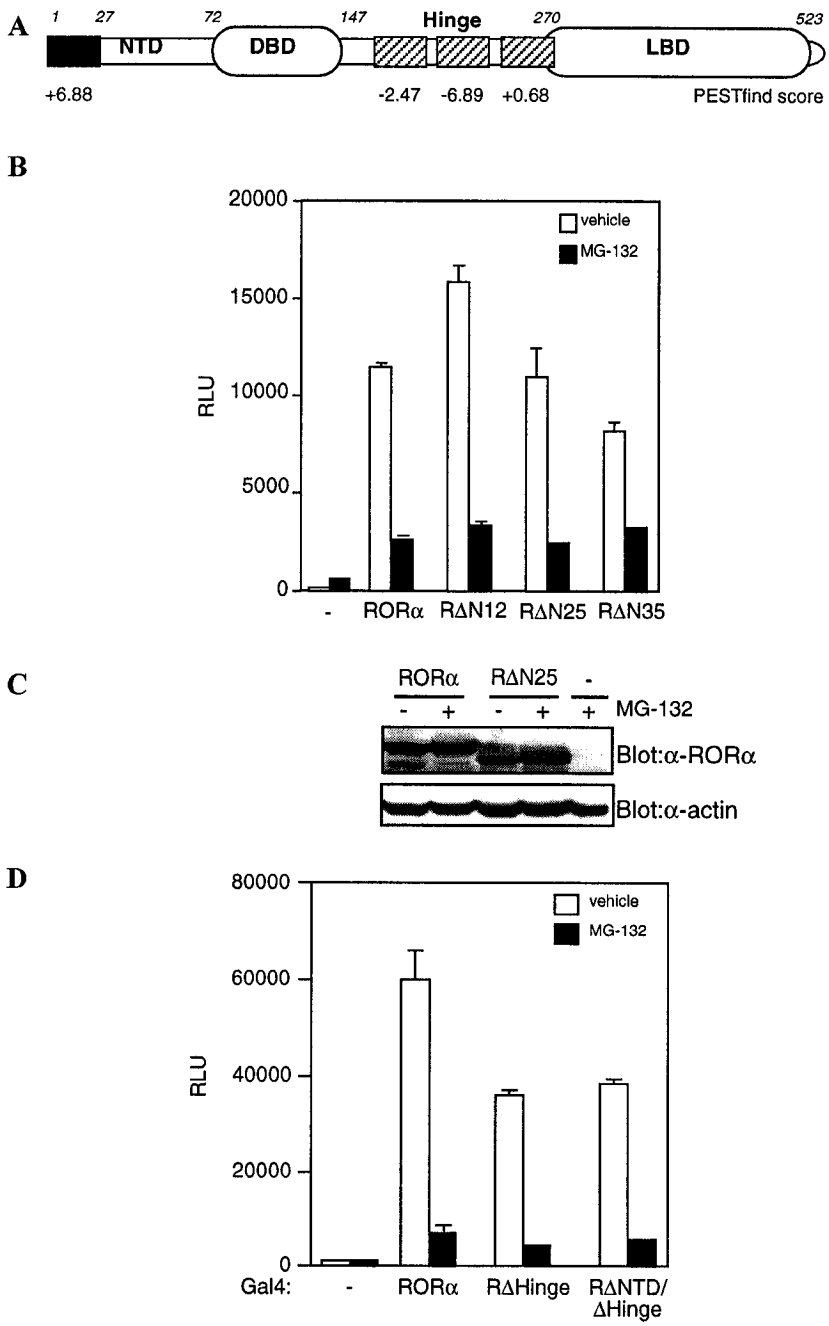
- + - + - + - + - + - +
Blot:α-RORα
Blot:α-actin

Figure 3. RORα degradation requires an intact ligand binding domain. (A) Cos-1 cells were transiently transfected with empty vector (control), wild type RORα, ligand binding pocket mutants ($ROR\alpha^{L361F}$ and $ROR\alpha^{V364G}$), and AF-2 mutants ($ROR\alpha^{K357A}$ and $ROR\alpha^{E509K}$). Cells were treated with ethanol (vehicle) or 1 μM MG-132 proteasome inhibitor. Transcriptional activity was measured from a $RORE\alpha2_3$-TkLuc reporter gene. Normalized values are calculated in terms of % RORα activity with respect to wild type. (B) Cell extracts assayed for transcriptional activity were subjected to immunoblotting with anti-RORα antibody (top panel) and anti-actin antibody (bottom panel), for detection of RORα wild type and mutant proteins, in cells treated with ethanol (-) or 1 μM MG-132 (+). Detection of actin serves as a control.

Figure 4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27
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DBD
LBD
+6.88
-2.47
-6.89
+0.68
PESTfind score
B
20000
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5000
0
RLU
vehicle
MG-132
-
RORα
RΔN12
RΔN25
RΔN35
C
RORα
RΔN25
-
- + - + +
MG-132
Blot:α-RORα
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D
80000
60000
40000
20000
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RLU
vehicle
MG-132
Gal4:
-
RORα
RΔHinge
RΔNTD/
ΔHinge

Figure 4. Putative PEST motifs are dispensable for signaling to the ubiquitin-proteasome complex. (A) Schematic representation of the RORα receptor. Four putative PEST motifs, identified using a *PESTfind* program, are shown. A potential motif located in the N-terminal region of RORα (black box) has a score of +6.88, there are three weak PEST motifs (box with hatched bars) identified in the hinge region, the last one overlapping slightly with the ligand binding domain with scores of –2.47, -6.89, and +0.68, respectively. (B) N-terminal deletion mutants of RORα resulting in constructs with a weak N-terminal PEST motif (RΔ12) or devoid of a N-terminal PEST (RΔ25 and RΔ35), were transiently transfected in Cos-1 cells treated with ethanol (vehicle) or 1 μM MG-132, and assayed for transactivation potential on a RORE$\alpha 2_3$-TkLuc reporter. (C) Protein expression of RORα and RΔ25 lysates treated with ethanol (-) or 0.5μM MG-132 (+) was analyzed by immunoblotting using the anti-RORα antibody. (D) Gal4-RORα fusion proteins encoding full length RORα, N-terminal deletion mutant (RΔNTD) and N-terminal and hinge region deletion mutant (RΔNTD/ΔHinge), encoding the weak PEST motifs only, or devoid of any PEST motifs, respectively. Transactivation potential of these Gal4-RORα fusion proteins were assayed from a UAS_2-TkLuc reporter from transiently transfected Cos-1 cells treated with ethanol (vehicle) or 1 μM MG-132.

Chapter V General Discussion

The RORα orphan nuclear receptor is a strong transcriptional activator that regulates the expression of genes essential for a number of cellular and physiological processes. RORα plays an important role in regulating genes involved in lipid and lipoprotein metabolism, muscle differentiation, bone metabolism, immunological responses, and neuronal differentiation (reviewed in Jetten et al., 2001). The RORα deficient mouse serves as a model for age-related degenerative diseases, given that the deregulation of these pathways potentially leads to atherosclerosis, muscular atrophy, osteoporosis, immunodeficiencies and cerebellar degeneration (reviewed in Jarvis et al., 2002). To date, RORα has been characterized as a constitutive transcriptional activator, potently inducing the expression of its target genes through recruitment of coactivator proteins in absence of exogenously added ligand (Atkins et al., 1999; Lau et al., 1999). Inhibition of RORα-mediated transcription has only been demonstrated to occur through passive repression, despite binding to the NCoR corepressor *in vitro* (Harding et al., 1997). A great deal of interest has been generated in understanding the mechanisms that regulate RORα transcriptional activity. In this study we have investigated the mechanisms involved in the regulation of RORα by studying the DNA binding mode, the recruitment of coactivator and corepressor proteins, and the ubiquitin-proteasome mediated degradation. In addition, the question of whether a putative RORα ligand exist and whether it regulates coregulator exchange or receptor proteolysis was also addressed.

1. DNA binding and Passive Repression

The DBD is truly the hallmark domain defining the nuclear receptor superfamily as sequence-specific transcription factors. Nuclear receptors recognize their hormone response elements either as monomers, homodimers, or heterodimers with the common partner RXR (Glass, 1994). *In vitro* mutagenesis studies, as well as crystal structures of several nuclear receptor DBDs complexed to DNA, have been instrumental in dissecting the molecular determinants involved in both protein-DNA and protein-protein interactions required for high affinity DNA binding (Danielsen et al., 1989; Luisi et al., 1991; Mader et al., 1989; Umesono and Evans, 1989). Evolutionarily, very subtle changes in the amino acid composition of this DNA recognition domain has resulted in the generation of nuclear receptors able to discriminate between two consensus hexameric sequences, namely the GR (AGAACA) and ER (AGGTCA) motifs, encoded in the regulatory regions of a myriad of genes (Mader et al., 1989). Similarly, it has been hypothesized that the primordial nuclear receptor is a monomeric binding protein from which dimeric receptors were generated by evolutionary changes. Subsequently, a great deal of flexibility has been introduced, allowing a given receptor to bind DNA both as a monomer and as a homodimer, exemplified by ERR, NGFI-B, and T_3R (Forman et al., 1992; Johnston et al., 1997; Philips et al., 1997; Sem et al., 1997; Vanacker et al., 1999; Wahlström et al., 1992). There are two dimerization interfaces in nuclear receptors, one located at the very C-terminal of the receptor in the LBD, and the second one in the DBD. In the absence of DNA, two subunits of a given receptor are brought into contact by the LBD dimerization interface. Positioning of the DBD with

respect to the particular arrangement of the tandem repeats, inverted versus direct for example, is made possible by the flexibility conveyed by the hinge region allowing the DBD to swivel into position. In order to accommodate dimerization on more than one type of repeat, plasticity in the amino acid residues involved in protein-protein interactions is required.

The ROR and Rev-ErbA receptors are two subfamilies of orphan nuclear receptors that share highly homologous DBDs, and regulate overlapping gene networks. While both receptors avidly bind DNA as monomers to a RORα response element (RORE), Rev-ErbAα also binds as a homodimer to an extended direct repeat (DR2) element (Dumas et al., 1994; Harding and Lazar, 1993; Retnakaran et al., 1994). RORα-mediated transcription is passively repressed by the Rev-ErbAα/RVR receptors, competing for binding to overlapping HRE regulating expression of a number of genes including the N-myc proto-oncogene, the apolipoprotein CIII gene, and the Rev-ErbAα gene itself (Coste and Rodriguez, 2002; Delerive et al., 2002; Dussault and Giguère, 1997). Rev-ErbAα/RVR are potent transcriptional repressors, constitutively bound to NCoR corepressor, they lack an AF-2 helix unable to recruit coactivators (Harding and Lazar, 1995). We used RORα and Rev-ErbAα as a model for the study of the molecular determinants involved in the transition from monomeric to homodimeric modes of DNA binding by nuclear receptors.

As previously demonstrated by our laboratory, monomeric binding is mediated by the highly conserved CTE which stabilizes the monomer by contacting bases of the 5'A/T rich flanking region preceding both an RORE or an extended DR2 (Giguère et al., 1995). The crystal structure of homodimeric Rev-ErbAα complexed to an

extended DR2 demonstrated that the CTE and residues of the second zinc finger module form the dimer interface (Zhao et al., 1998). The CTE therefore has the dual role of mediating both protein-DNA and protein-protein interactions. This region is conserved between RORα and Rev-ErbAα, suggesting that progressive evolutionary changes were made in the zinc finger modules to achieve homodimer binding. Interestingly, these are also conserved in RORα, with the exception of one key isoleucine residue at the very tip of the second zinc finger module in Rev-ErbAα. We demonstrated in this study that a point mutation substituting a threonine for an isoleucine residue in RORα, allows this orphan receptor to form dimers. Although dimerization of this point mutant was not as efficient as Rev-ErbAα, suggesting that additional residues are involved.

A complete transition to homodimeric binding required an additional three amino acid change within the first zinc finger of RORα. Although, equivalent residues in Rev-ErbAα have not been directly implicated in the dimer interface, based on the crystal structure, they may be involved in mediating intramolecular interactions required for the proper positioning of those forming the dimer interface (Zhao et al., 1998). Interestingly, only the ROR family and its *Drosophila* and *C. elegans* orthologs encode a residue with a hydrocarbon side chain at position 88 of the first zinc finger. All other dimeric binding receptors encode an amino acid residue with an aromatic ring at this position. The specific side chain of the residue occupying this position may serve as an indicator predicting the DNA binding mode of a given nuclear receptor (Moraitis and Giguère, 1999).

We have demonstrated that the transition from monomeric binding to homodimeric binding can be achieved with relatively very few changes in the DBD. This complements previous work from our laboratory, demonstrating that the NTD, the hinge domain and the DBD all work in concert mediating high affinity and specific DNA-binding properties. Furthermore, the NTD and the hinge region were shown to orient the zinc finger modules and the CTE relative to each other in order to achieve proper DNA binding, concurrently influencing DNA bending against which the RORα receptor could be tightly nestled (Giguère et al., 1995; Giguère et al., 1994; McBroom et al., 1995).

2. Ligand-Dependent Transcriptional Activation

The molecular determinants governing RORα DNA binding have been carefully investigated. We therefore studied the mechanisms that mediate RORα constitutive transcriptional activity once it is recruited to its HRE. A major controversy in the nuclear receptor field was the claim that the melatonin hormone is the natural ligand for RORα (Becker-André et al., 1994). This was dismissed given the irreproducibility of the data, and it is now clear that RORα in a cell-based assay activates reporter gene transcription in the absence of any exogenously added ligand (Greiner et al., 1996; Tini et al., 1995). Nevertheless, the possibility of the existence an unidentified intracrine ligand regulating this potent transcriptional activator cannot be overlooked.

In an effort to determine whether RORα activity is ligand-dependent, we introduced a series of mutations in the ligand binding domain. Mutagenesis of conserved amino acids in the pocket led to a decrease of RORα activity, suggesting

that binding of a putative ligand is impaired and supporting an endogenous-ligand model. The AF-2 helices of RORβ and RARα have been shown to be functionally interchangeable (Greiner et al., 1996). We generated a chimeric RARα, encoding the AF-2 helix of RORα (RARα-R), yielding a functional receptor responsive to retinoic acid, interacting with SMRT corepressor and SRC-1 coactivator in a ligand-independent and ligand-dependent fashion, respectively. This demonstrated that the RORα AF-2 helix is functional in the context of a liganded-receptor, lending further support to the model of an intracrine RORα ligand regulating the transcriptional activity of this receptor.

A putative RORα ligand would have to parallel the ubiquitous expression of the receptor. Alternatively, RORα may recognize a number of structurally related low affinity compounds, expressed in different cell types. This is not uncommon, given that a number of orphan nuclear receptors have been shown to accommodate more than one type of structurally related molecules in their ligand binding domains. For example, LXR binds oxidized derivatives of cholesterol, oxysterols, and regulates their metabolism, serving as a cholesterol sensor (Janowski et al., 1996; Lehmann et al., 1997). Similarly, FXR has been shown to be a receptor for bile acids enhancing the expression of genes such as the intestinal bile cid binding protein, and inhibiting CYP7A involved in bile acid synthesis (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Moreover, PXR is a steroid and xenobiotic sensor that binds structurally unrelated xenobiotic compounds, and increases the expression of the detoxification cytochrome P450 genes (reviewed in Giguère, 1999; Kliewer et al., 2002; Kliewer et al., 1999; Lehmann et al., 1998). RORβ expression is restricted to

the brain and the central nervous system, and thus likely binds a neuronal-specific ligand. Similarly, RORγ expression is also limited and given its role in lymph node organogenesis and thymopoiesis, it may be regulated by a ligand with a specific role in the immune system. Identification of a natural RORα ligand would provide a powerful means of defining the physiological role of this orphan. There are three approaches that have been useful in identifying putative orphan ligand (Willson and Moore, 2002). The first approach involves screening a selected set of ligands based on the pathways that are regulated by a given receptor. Determination of specific cellular and metabolic pathways regulated by RORα will provide important knowledge regarding the type of lipophilic molecule that may be recognized by this orphan. The second approach is to randomly screen known drug molecules for binding. Identification of a synthetic molecule binding to RORα could potentially provide important information regarding the structure of a candidate RORα ligand, extrapolated towards the identification a natural ligand. The third approach is X-ray crystallography of the RORα ligand binding domain, which may result in entrapment of either a *bone fide* ligand or a fortuitous ligand, which reveals important clues as to the structure of a natural ligand, which may narrow the search of candidate molecules. The challenge of crystallizing the LBD of an orphan receptor is much greater than one for which the ligand is known and is used to stabilize the structure. Crystallization of the RORβ LBD required the addition of a SRC-1 peptide, encoding the LxxLL motif, shifting the equilibrium towards a more stable agonist-conformation (Stehlin et al., 2001). Mass spectroscopy analysis identified a fortuitous ligand, stearic acid, occupying a low percentage of the ligand binding pocket, in a

partially disordered conformation. Stearic acid was unable to activate RORβ in a cell-based reporter assay, therefore did not meet the criteria of a *bone fide* ligand. Trapping of a fortuitous ligand during the crystallization process is not uncommon, and has also been observed with the RXRα and USP receptors where oleic acid and a phospholipid occupied their LBPs, respectively (Billas et al., 2001; Bourguet et al., 2000). Moreover, despite its inability to activate the receptor, a fortuitous ligand is able to stabilize the receptor in an active conformation, mimicking an important property of a *bone fide* ligand.

Recently, the crystal structure of RORα was solved revealing that cholesterol (cholest-5-en-3β-ol) is a putative ligand (Kallen et al., 2002). Interestingly, this also confirmed our hypothesis of RORα being a ligand-dependent receptor regulated by a relatively ubiquitous molecule, which explains the constitutive activity observed for this orphan. Moreover, it was demonstrated that cholesterol bound to RORα can be exchanged and that intracellular cholesterol modulates RORα transcriptional activity, therefore suggesting that this orphan receptor may play a role in the regulation of cholesterol homeostasis. Given that RORα deficient mice display severe atherosclerosis and hypolipoproteinemia, we could hypothesize that RORα modulates genes whose role is to control intracellular cholesterol levels (Mamontova et al., 1998). Future studies are required to confirm that cholesterol or a cholesterol derivative is indeed the physiological ligand for RORα, and to rule out the possibility that these effects are indirect as a result of an overall change in cellular levels of cholesterol. For example, ligand binding affinity studies would be required to measure the association and/or dissociation constant of cholesterol for the RORα

LBD, in order to determine whether physiological concentrations of cholesterol could feasibly modulate RORα activity. Moreover, given that ligand binding regulates coregulator recruitment, it would be important to assay the interaction of corepressors and coactivators with RORα in presence of cholesterol using GST pull-down or co-immunoprecipitation assays.

Ligand binding tightly regulates the interaction with coregulator proteins by inducing the transconformation of the LBD and mediating coregulator exchange. In this study, we have demonstrated that mutation of the coactivator binding interface, formed by residues of the hydrophobic cleft and the AF-2 helix, abolishes RORα transcriptional activity due to an impaired ability to recruit SRC coactivator proteins. It has been demonstrated that RORα recruits components of the activation, co-integrator, and mediator complexes, bringing histone remodeling and histone acetyltransferase activities to the promoter of its target genes (Atkins et al., 1999; Lau et al., 1999). In light of our data, this is probably mediated in a ligand-dependent fashion, suggesting that complexes required for transcriptional activity are shared among nuclear receptors. Specificity is therefore dictated by the response element of a given gene, and the presence of a specific ligand inducing transcription through its cognate receptor.

3. Transcriptional Repression by a Ligand-Oblivious Corepressor

Downregulation of RORα transcriptional activity has been successfully demonstrated to occur via a passive repression mechanism, whereas active repression of RORα-target gene transcription has not been as obvious prior to this study. Like

many members of the nuclear receptor superfamily, RORα also interacts with NCoR and SMRT *in vitro*, but this interaction is inhibited by the AF-2 helix when the receptor is bound to DNA, and efficient repression by NCoR/SMRT of RORα-mediated repression has not been demonstrated (Harding et al., 1997). Here, we have demonstrated that Hr potently represses the transcriptional activity of all three members of the ROR subfamily. This novel corepressor is a T_3R-specific binding protein, whose gene expression is regulated in the cerebellum in a thyroid hormone-responsive fashion (Thompson and Bottcher, 1997). Despite its lack of sequence identity with NCoR and SMRT corepressors, Hr mediates transcriptional repression in a manner analogous to these classic corepressors, through recruitment of histone deacetylases (Potter et al., 2001). Similarly, the Hr-RORα interaction is considerably enhanced in absence of the AF-2 helix *in vitro*. In sharp contrast to other corepressor-nuclear receptor interactions, Hr binding to RORα is mediated by two LxxLL-containing motifs, a mechanism that has generally been associated with coactivator interaction. Remarkably, the specificity of Hr corepressor action can be transferred to RAR by exchanging the AF-2 helix. Repression of the chimeric RARα-R was observed in the presence of retinoic acid, demonstrating that in this context, Hr is a ligand-oblivious nuclear receptor corepressor. Moreover, given that RORα is regulated by an endogenous ligand, and Hr utilizes coactivator-like leucine rich motifs, we suspected that this interaction may occur with agonist-bound RORα. These results suggest a novel molecular mechanism for corepressor action and demonstrate that the AF-2 helix can play a dynamic role in controlling corepressor as well as coactivator interactions.

In vitro, the RORα LBD is in an active conformation favoring interaction with SRC coactivators, and exerts an inhibitory influence on Hr binding. This suggests that the AF-2 helix masks the molecular determinants required for Hr interaction, that are otherwise unveiled *in vivo*. We could hypothesize that post-translational modification or a tertiary protein may be involved in anchoring the AF-2 helix in a conformation that is favorable for Hr binding. It has previously been demonstrated that the presence of Ca^{+2}/calmodulin-dependent protein kinase IV potentiates RORα transcriptional activity not by directly phosphorylating the receptor, rather by enhancing its interaction with peptides encoding LxxLL motifs (Kane and Means, 2000). This may also hold true for the interaction observed between RORα and Hr, which may be required to obtain a stable protein-protein interaction, and would explain the differences *in vitro* and in a cell-based assay. Alternatively, a tertiary protein may act as a bridging factor, interacting simultaneously with both RORα AF-2 helix and Hr. Given that corepressor interaction with nuclear receptors involves recruitment of a complex of proteins with enzymatic activities, it would be of interest to identify the tertiary proteins or any others involved in Hr-mediated repression. A yeast three-hybrid assay would be a useful method of identifying novel proteins that are essential in forming a functional ROR-Hr complex. In addition, components of a putative ROR-Hr complex could be identified using the tandem affinity purification method (Puig et al., 2001). Our study demonstrates that the AF-2 helix dictates more than coactivator binding, providing specificity for corepressor binding as well. Similarly, coregulators may also be more flexible than the classical NCoR/SMRT corepressors

and SRC coactivators, utilizing interchangeable mechanisms to mediate nuclear receptor interaction.

4. Cross-talk Between RORα, Rev-ErbAα and T_3R pathways

Thyroid hormone and RORα-mediated pathways play a critical role in cerebellar development. Interestingly, RORα deficient mice and hypothyroid mice share similar phenotypic traits, namely exhibit morphological abnormalities of Purkinje cells (Bouvet et al., 1987; Dussault et al., 1998; Hamilton et al., 1996; Matysiak-Scholze and Nehls, 1997). Hr is the molecular link converging the T_3R and RORα pathways. Hr likely acts as a developmental and tissue-specific inhibitor of RORα orphan receptor, providing a means of hormonally regulating RORα transcriptional activity. Thyroid hormone can thereby indirectly control the timing and intensity of RORα activity through regulation of the spatio-temporal expression of Hr (Figure 1). Moreover, Purkinje cells do not express Hr corepressor allowing the receptor to function optimally given the importance of RORα for the survival of these cells.

Silencing RORα activity in these neuronal cells likely does occur by passive repression through the cross talk of RORα and Rev-ErbAα pathways. Interestingly, Rev-ErbA$\alpha^{-/-}$ mice also display cerebellar defects, due to morphological abnormalities of both Purkinje cells and granule cells, namely delayed proliferation and migration of these cells. Although, unlike *staggerer* mice, Rev-ErbAα deficient mice are not severely ataxic (Chomez et al., 2000). Interestingly, Rev-ErbAα expression is induced by RORα via an RORE element encoded in its promoter region (Delerive et al., 2002).

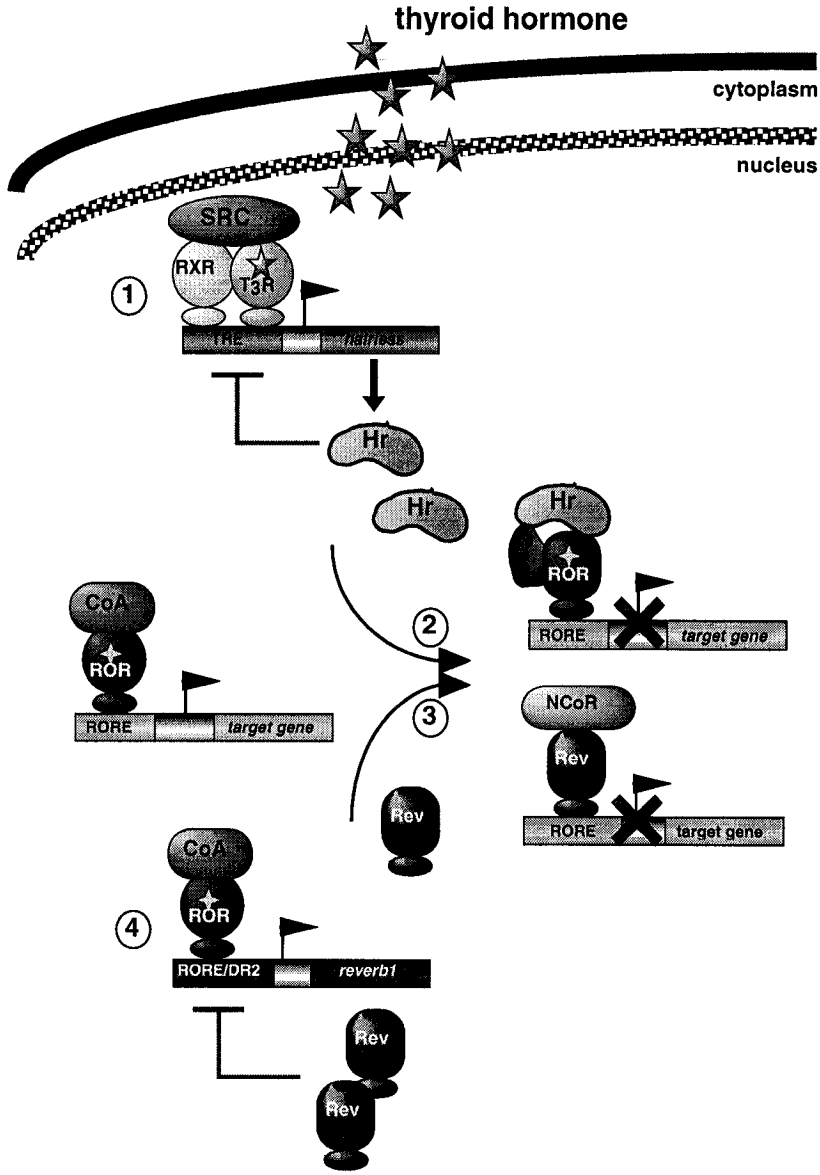

Figure 1. Schematic representation of the potential cross-talk between the T_3R, RORα and Rev-ErbAα receptors. Thyroid hormone stimulation leads to the upregulation of the *hr* gene. Hr corepressor inhibits T_3R activity (1), autoregulating its own transcription and is a RORα corepressor (2), providing a means to control RORα activity in response to thyroid hormone. RORα and Rev-ErbAα receptors oppositely regulate the same target genes (3). Among which is the *reverbA* gene. RORα upregulates the expression of Rev-ErbAα through an RORE element, whereas Rev-ErbAα represses its own transcription via a DR2 element (4).

This generates an autoregulatory feedback loop, where RORα-induced expression of Rev-ErbAα is competed by Rev-ErbAα, inhibiting its own transcription (Figure 1). Rev-ErbAα expression occurs at a later stage of Purkinje cell development, therefore passively repressing RORα-target genes. For example, the Purkinje cell specific protein-2 (pcp-2) gene is oppositely regulated by RORα and Rev-ErbAα, and can also be regulated by T_3R. The function of this gene in the neuronal cells of the cerebellum remains elusive (Chomez et al., 2000; Matsui, 1997; Zou et al., 1994).

5. Ubiquitin-Proteasome Complex Mediated Regulation

Coactivators and corepressors are part of large complexes of proteins, tethered to nuclear receptors. As previously discussed, activation complexes contain proteins with various enzymatic activities, including histone acetylases, methylases as well as enzymes with ubiquitin-ligase activity. Proteasome-mediated degradation has been closely coupled to transcriptional activation, providing a fail-safe mechanism against deleterious levels of transcription. The ubiquitin-proteasome complex plays an integral role in transcription, and is emerging as a key player of mRNA synthesis (Laroia et al., 1999). This complex is part of the transcriptional machinery, recruited to the promoter by transcription factors, the activation complex, as well as by the RNA polymerase II (reviewed in Tansey, 2001; Thomas and Tyers, 2000). Nuclear receptor turnover is generally ligand-dependent and mediated by the ubiquitin-proteasome complex (reviewed in Dennis et al., 2001). In this study, we demonstrated that the ubiquitin-proteasome pathway regulates RORα stability. RORα is ubiquitin-conjugated and targeted for destruction by the 26S proteosome. Degradation is

blocked by the pseudosubstrate MG-132, an inhibitor of the proteasomal catalytic subunit. Interestingly, RORα transcriptional activity is compromised when the 26S proteasome is inhibited, suggesting that a functional ubiquitin-proteasome pathway is required for transcriptional activation. Similarly, the transcriptional activities of a number of nuclear receptors are abolished upon blocking of this pathway. The intrinsic instability of many nuclear receptors permits a rapid turnover, which allows the cell to rapidly re-initiate transcription in response to a new round of hormonal stimulation. A drawback in studying orphan receptors is the lack of an identified ligand. To overcome this obstacle, we utilized ligand binding pocket mutants to assess the role of a putative ligand in this ubiquitin-mediated RORα degradation. Impairing ligand binding as well as coactivator binding resulted in an increased stability, suggesting that a functional RORα receptor is imperative for proteolysis. Activation domains are often linked to degradation signals, or motifs recognized by E3 ubiquitin ligases, triggering the demise of the protein by the proteosome complex. An inverse correlation has been established between the potency of a given activation domain and the rate at which the protein is degraded (Molinari et al., 1999; Salghetti et al., 2001; Salghetti et al., 2000). Many proteasomal targets contain regions encoding PEST motifs (Rechsteiner and Rogers, 1996; Rogers et al., 1986). Although, putative PEST motifs are encoded in RORα, they are not involved in signaling the degradation of the receptor. A functional coactivator interface is therefore essential not only for the recruitment of an activation complex, but also for the recruitment of proteins involved in the ubiquitin-proteosome complex. The ubiquitin ligases, E6-AP and RPF1, and a component of the 19S subcomplex, SUG-1,

have been shown to also function as nuclear receptor coactivators (Gottlicher et al., 1996; Imhof and McDonnell, 1996; Lee et al., 1995; Nawaz et al., 1999; Rubin et al., 1997; von Baur et al., 1996). Interestingly, RORα LBD was demonstrated to recruit SUG-1, although the functional significance of this interaction remained elusive (Atkins et al., 1999). We hypothesize that binding of SUG-1 by RORα leads to recruitment of the ubiquitin-proteasome complex, results in receptor downregulation. Interestingly, the ubiquitin-proteasome pathway regulates RORα transcriptional activity, where blocking this pathway also results in inhibition of RORα-mediated transcriptional activity, demonstrating that these two events are closely coupled. Degradation of nuclear receptors and components of the activation complex provides a means of freeing of the response element allowing a new round of transcription to begin. In addition, transcription factor degradation may be initiated by the RNA polymerase II arising from recruitment of E3 ubiquitin ligases signaled by phosphorylation of the C-terminal domain of the polymerase, freeing the promoter of the pre-initiation complex and allowing elongation to occur (reviewed in Thomas and Tyers, 2000).

6. Conclusion

As evidence from our study as well as those of others is pieced together, the model for RORα-mediated transcriptional activation is beginning to unravel. In general, transcriptional regulation by RORα is controlled by the concerted action of coactivator and corepressor proteins, activating and repressing respectively, through mutually exclusive interactions, a mechanism shared by most members of the nuclear

receptor superfamily. Interestingly, the precise mechanisms of both DNA and protein interactions sets RORα apart from the those described for classical nuclear receptors such as RARα, T_3R or ER. RORα is strictly a monomeric DNA binding protein unable to form homodimers or heterodimers with any other nuclear receptor studied to date. This orphan receptor is a potent transcriptional activator that is ubiquitously expressed and is transcriptionally active in almost every cell type and has therefore been described as a constitutive activator. We provide evidence that this activity is due to an endogenous ligand, suggesting that a putative RORα ligand would also have to be ubiquitously expressed in order for constitutive activity to be observed. It has been suggested that cholesterol or a cholesterol derivative may be the physiological ligand for RORα, but further studies are required to confirm this molecule as its *bone fide* ligand (Kallen et al., 2002). Cell specific regulation of RORα-mediated transcription is therefore dependent on the presence of cell type specific coregulator proteins indirectly controlling the expression of RORα target genes. Strinkingly, the AF-2 helix of RORα plays a dynamic role in transcriptional activation by dictating specificity for both SRC coactivator and Hr corepressor binding. Interestingly, Hr-mediated repression of RORα transcriptional activity occurs in presence of ligand, in stark contrast to the mechanism described for NcoR/SMRT corepressors whose interactions with nuclear receptors is abolished in presence of ligand. In addition to active repression mediated by Hr, silencing of RORα transcriptional activity also occurs by passive repression involving displacement of RORα from its response element by RevErbA family members. Finally, the cell is protected against deleterious levels of RORα-mediated transcriptional activation by the ubiquitin-

proteasome pathway, which regulates the stability of the receptor, limiting promoter occupancy by this potent activator (Figure 2).

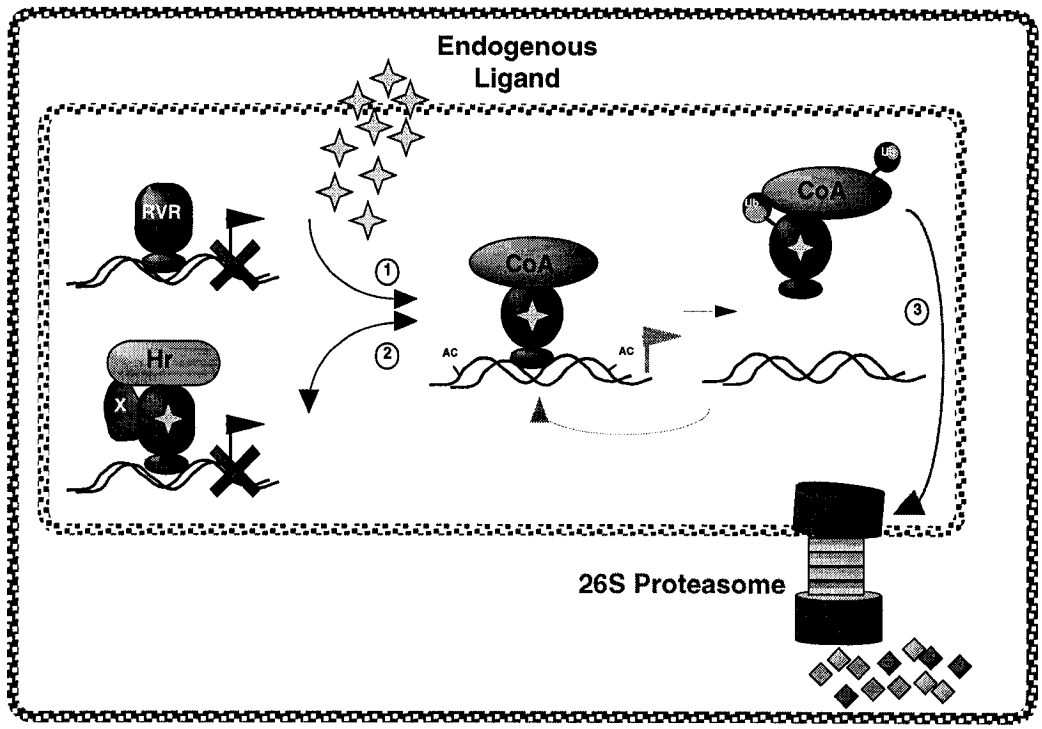

Figure 2. Model depicting the regulation of RORα-mediated transcription. RORα transcriptional activity is dependent on binding of an endogenous ligand and coactivator binding (CoA), essential for the recruitment of the histone acetylases, unwinding chromatin, and the assembly of the pre-initiation complex. This transcriptional activity is regulated by three mechanisms: (1) Passive repression mediated by Rev-ErbAα/RVR, competing for the same response element. (2) Active repression mediated by the ligand-oblivious Hr corepressor. Hr:RORα interaction likely involves a ternary partner (X), anchoring the AF-2 helix and unmasking determinants required for Hr binding. (3) Degradation by the ubiquitin-proteasome complex mediating RORα turnover, freeing the promoter allowing for transcriptional re-initiation to occur.

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Chapter VI. Contribution to Original Research

1. RORα is strictly a monomeric binding orphan nuclear receptor, lacking a functional dimerzation interface in its DBD.

2. There are four key dimerization determinants in the Rev-ErbAα DBD, sufficient to confer RORα with the ability to form cooperative homodimer complexes.

3. RORα transcriptional activity is dependent on the integrity of the LBD:

(A) Mutations in the LBP leads to a loss of transcriptional activity, suggesting that it is a ligand-dependent orphan receptor.

(B) Mutation in the AF-2 domain abolishes RORα activity, as well as recruitment of members of the SRC coactivator family.

4. The AF-2 helix dictates both coactivator and corepressor interactions.

5. ROR-mediated transcription is actively repressed by the Hr, a ligand-oblivious corepressor that is recruited in an AF-2 specific fashion.

6. Hr interaction with RORα is mediated by LxxLL motifs, a mechanism generally associated with coactivators, although Hr does not compete for the same molecular determinants at the surface of the RORα LBD.

7. RORα turnover is mediated by the ubiquitin-proteasome pathway, given that RORα can be ubiquitin-conjugated *in vitro*, and blocking of the 26S proteasome leads to an increase in RORα protein expression.

8. Ligand and coregulator binding perpetuate RORα degradation. RORα LBD mutants abolishing both these functions display greater protein stability, relative to wild type receptor.

9. Inhibition of the ubiquitin-proteasome pathway also leads to a loss of RORα transcriptional activity, suggesting that transcription and proteasomal degradation are closely linked events.

10. RORα transcriptional activity is regulated by an endogenous ligand, recruitment of SRC coactivators, Hr corepressor, and by the ubiquitin-proteasome pathway.

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