The ROR Nuclear Orphan Receptor Subfamily: Critical Regulators of Multiple Biological Processes

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The nuclear receptor superfamily, a group of structurally related, ligand-dependent transcription factors, includes a large number of orphan receptors for which no ligand has yet been identified. These proteins function as key regulators of many physiological processes that occur during embryonic development and in the adult. The retinoid-related orphan receptors (RORs) α, β, and γ comprise one nuclear orphan receptor gene subfamily. RORs exhibit a modular structure that is characteristic for nuclear receptors; the DNA-binding domain is highly conserved and the ligand-binding domain is moderately conserved among RORs. By a combination of alternative promoter usage and exon splicing, each ROR gene generates several isoforms that differ only in their amino terminus. RORs bind as monomers to specific ROR response elements (ROREs) consisting of the consensus core motif AGGTCA preceded by a 5-bp A/T-rich sequence. RORE-dependent transcriptional activation by RORs is cell type–specific and mediated through interactions with nuclear cofactors. RORs have been shown to interact with certain corepressors as well as coactivators, suggesting that RORs are not constitutively active but that their activity is under some regulatory control. RORs likely can assume at least two different conformations: a repressive state, which allows interaction with corepressor complexes, and an active state, which promotes binding of coactivator complexes. Whether the transition between these two states is regulated by ligand binding and/or by phosphorylation remains to be determined. Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) can dramatically enhance ROR-mediated transcriptional activation. This stimulation involves CaMKIV-mediated phosphorylation not of RORs, but likely of specific nuclear cofactors that interact with RORs.

RORα is widely expressed. In the cerebellum, its expression is limited to the Purkinje cells. ROR$\alpha^{-/-}$ mice and the natural RORα-deficient *staggerer* mice exhibit severe cerebellar ataxia due to a defect in Purkinje cell development. In addition, these mice have thin long bones, suggesting a role for RORα in bone metabolism, and develop severe atherosclerosis when placed on a high-fat diet. Expression of RORβ is very restricted. RORβ is highly expressed in different parts of the neurophotoendocrine system, the pineal gland, the retina, and suprachiasmatic nuclei, suggesting a role in the control of circadian rhythm. This is supported by observations showing alterations in circadian behavior in ROR$\beta^{-/-}$ mice. RORγ, which is most highly expressed in the thymus, plays an important role in thymopoiesis. Thymocytes from ROR$\gamma^{-/-}$ mice undergo accelerated apoptosis. The induction of apoptosis is, at least in part, due to a down-regulation of the expression of the antiapoptotic gene Bcl-X_L. In addition to the thymic phenotype, ROR$\gamma^{-/-}$ mice lack lymph nodes, indicating that RORγ is essential for lymph node organogenesis. Overexpression of RORγ has been shown to inhibit T cell receptor–mediated apoptosis in T cell hybridomas and to repress the induction of Fas-ligand and interleukin 2. These studies demonstrate that RORs play critical roles in the regulation of a variety of physiological processes. Further characterization of the mechanisms of action of RORs will not only lead to the identification of ROR target genes and provide additional insight into their normal physiological functions, but will also determine their roles in disease.

I. Introduction

The nuclear hormone receptor superfamily consists of structurally related, ligand-dependent transcription factors (*1–4*). This family includes receptors for steroid hormones, retinoic acid, thyroid hormone, vitamin D_3, eicosanoids, and bile acids (*1, 5–7*). In addition, a large number of genes have been cloned, that encode orphan receptors, receptors for which regulatory ligands have not yet been identified.

Nuclear receptors share a common modular structure composed of several domains: the amino-terminal domain (A/B region), DNA-binding domain (DBD or C region), the ligand-binding domain (LBD or E region), and a flexible hinge domain (D region) connecting the DBD and LBD (*1, 4, 8*). Some receptors contain an extensive carboxyl-terminal domain for which a function has not yet been clearly established. In certain receptors, the amino-terminal domain contains a ligand-independent transactivation function (AF-1). This domain can also influence the affinity with which receptors bind DNA elements (*9*). The DBD, which is the most highly conserved region among nuclear receptors, targets the receptor to specific DNA sequences known as hormone response elements (REs). These REs are usually located in the upstream promoter region of target genes. The DBD encompasses two "zinc-finger" motifs, each containing an α-helix referred to as a P- or D-box. The P-box makes specific base contacts between the receptor and the major groove of the DNA helix, while the D-box is involved in protein–protein interactions, particularly in homo- and heterodimerization of nuclear receptors. The carboxyl-terminal extension (CTE), a region adjacent to the DBD, is also highly conserved among members of each nuclear receptor subfamily; this region influences the RE-binding affinity of the receptor. The nonconserved hinge domain can have multiple functions in repression and activation. The LBD combines several important functions. In addition to forming a ligand-binding pocket, it contains regions that are critical in repression, activation, nuclear localization, and dimerization (*4, 7*). In certain receptors (e.g., the estrogen receptor) the LBD is involved in interactions with heat-shock proteins (*10*).

Analysis of the crystal structure of the LBD of several receptors revealed a very similar canonical structure consisting of 11–12 helical regions (*11, 12*). Helices 3–5 play an essential role in the transcriptional regulation by nuclear receptors in that they provide the interaction surface for several coactivators and corepressors. Helix 12 contains the core motif of the transactivation function 2 (AF-2) and is critical in the control of transcriptional activity of nuclear receptors. The conformation of the agonist-bound (holo) receptor has been reported to differ significantly from that of the unliganded (apo) receptor. For example, in retinoid and PPAR receptors (*11, 12*), ligand binding induces an extensive shift in the position of helix 12, resulting in the dissociation of a multimeric corepressor

complex that consists of corepressors, histone deacetylases, and other cofactors (*13–15*). This conformational change promotes the formation of a large multimeric coactivator complex containing coactivators, histone acetylases, and additional cofactors (*11–13, 15, 16*). The latter enzymes cause acetylation of nucleosomal histones and local remodeling of chromatin structure. Interaction of these multimeric complexes with the basal transcriptional machinery results in the activation of RNA polymerase II and enhanced transcription of target genes. However, nuclear receptors can influence gene expression by a number of other mechanisms, such as the inhibition of NF-κB or AP-1–mediated transcription by glucocorticoid and retinoid receptors (*17, 18*).

Members of the nuclear hormone receptor superfamily have been reported to regulate a variety of physiological processes, including many aspects of embryonic development, differentiation, proliferation, homeostasis, and metabolism (*3, 5–7, 19, 20*). Genetic alterations and changes in the expression of several receptors have been implicated in a number of pathological conditions (*5, 6, 21, 22*). Identification of natural and synthetic agonists and antagonists has made it possible to interfere in normal as well as pathological processes and has led to novel strategies in drug development and new therapies for a variety of illnesses, including cancer and diabetes (*22–24*).

The retinoid-related orphan receptors (RORs) α, β, and γ, initially referred to as RZRs and named NR1F1, -2, and -3, respectively, by the Nuclear Receptor Nomenclature Committee, constitute one subfamily of nuclear orphan receptors (*9, 25–28*). In this chapter, we analyze and compare the structure, mechanism of action, and functions of this subfamily of nuclear receptors.

II. Cloning and Expression Pattern of RORs

A. RORα

ROR receptors were identified as a result of different strategies to clone novel members of the nuclear receptor superfamily. Nuclear receptors are particularly highly conserved in the two zinc fingers of the DBD. Using two degenerate primers, the sequences of which were based on the two most highly conserved DBD regions, and a template of poly(A)$^+$ RNA from a variety of tissues, PCR amplification has led to the cloning of DBDs of many novel orphan receptors, including RORs (*3, 8, 25, 27, 29*). 5′-RACE and cDNA library screening have subsequently been used to obtain their respective, full-length coding regions. hRORα, initially referred to as hRZRα, was the first member of the ROR subfamily to be cloned in this way from the RNA of human umbilical vein endothelial cells (*25*). Several cDNAs encoding multiple isoforms of hRORα were isolated by screening human retina and testis λgt11cDNA libraries (*30*). Four different RORα RNA species (α1–4) have been identified

in humans, while in mice only two isoforms, $\alpha 1$ and $\alpha 4$, have been detected (*30, 31*). These isoforms share the same DBD, hinge, and LBD regions but display different amino-terminal domains. These isoforms, which are generated by a combination of alternative promoter usage and exon splicing, have been reported to differ in their DNA-binding specificities and pattern of expression, and therefore regulate different physiological processes and target genes (*30*).

RORα mRNA has been detected in many tissues, including heart, brain, skin, muscle, lung, spleen, testis, ovary, thymus, and peripheral blood leukocytes (*25*). Peripheral blood leukocytes contain the highest level of RORα mRNA. In most tissues the predominant transcript is about 15 kb. Some tissues, including lung, testis, liver, and leukocytes, contain additional transcripts, 7.5, 5.5, and 2.3 kb in size, which may be generated by the use of alternative polyadenylation signals. Most mouse tissues, including skin, lung, kidney, thymus, and leukocytes, contain only RORα4 transcripts, while RORα2 and -α3 mRNA are exclusively detected in testis (*31, 32*). Mouse cerebellum, where RORα mRNA localizes only to the Purkinje neuronal cells, expresses both RORα1 and RORα4 transcripts. These cells arise from the proliferative zone above the fourth ventricle beginning on day 13 of murine embryogenesis and migrate along the glia from day 14 through 17. *In situ* hybridization of sections of E14 embryos revealed high expression of RORα in Purkinje precursor cells in the cerebellar anlage (*33*). The ataxia displayed by RORα-deficient mice is related to abnormalities in Purkinje cell differentiation (*32, 33*). RORα mRNA is also expressed in the thalamus and in the suprachiasmatic nuclei of the hypothalamus.

In the testis, RORα expression is observed only after sexual maturation and is localized specifically to the peritubular cells (*32*). Expression of RORα is also observed in the epithelial layer of the epididymus. In the skin, RORα is localized to the hair follicles, epidermis, and sebaceous glands. In the growing hair follicle (anagen stage) RORα expression is restricted to a discrete set of differentiating keratinocytes. Similarly, RORα is expressed in the differentiated, suprabasal layers of the epidermis. The latter indicates a role for RORα in the regulation of gene expression during epidermal differentiation.

B. RORβ

RORβ was originally cloned using a similar PCR strategy with RNA isolated from rat brain (*26*). RORβ mRNA expression is much more restricted than that of RORα and is most abundant in brain, pineal gland, and eye (*34*). *In situ* hybridization studies showed that RORβ mRNA expression localizes particularly to several regions of the central nervous system (*34, 35*). RORβ mRNA has been detected in the nonpyramidal neurons of layer IV and V of the cerebral cortex and is most highly expressed in primary sensory cortices, particularly the primary visual, auditory, somatosensory, and motor cortex. In the hypothalamus RORβ

mRNA was found to be most abundant in the suprachiasmatic nuclei. RORβ could not be detected in the hippocampus, striatum, cerebellum, the ventral part of the spinal cord, or the motor nuclei of the cranial nerves. In the spinal cord, RORβ localizes to layers of the dorsal horn that receive sensory input from the periphery (*35*). Developmental regulation of RORβ has been observed in the adenohypophysis (Rathke's pouch) in which RORβ is expressed highly during early development but at a low level in the adult; the reverse is true for the cerebral cortex (*35*). *In situ* hybridization has localized RORβ mRNA to the retina (in the retinal photoreceptor layer) and to the pineal gland, the principal site of melatonin synthesis. In the retina, the expression of RORβ in the inner and outer nuclear layer is highly regulated during development.

Thus far, two different isoforms, RORβ1 and -β2, have been identified which are likely derived by transcription from alternative promoters (*36*). The two RORβ proteins differ only in their amino-terminal sequence and exhibit a different pattern of expression. Expression of RORβ2 is restricted to the pineal gland and retina, while RORβ1 is expressed highly in cerebral cortex, hypothalamus, and thalamus, and at low levels in the pineal gland and retina. RORβ2 mRNA expression in the pineal gland and retina has been reported to oscillate dramatically and to change as a function of the circadian rhythm (*35, 37*). Pineal glands from daytime animals contain the 10-kb RORβ1 mRNA transcript, while the pineal gland from nocturnal animals also express the 1.5-kb RORβ2 mRNA transcript (*36*). RORβ expression does not change in the suprachiasmic nuclei or elsewhere (*35*). RORβ expression in the pineal gland has been reported to be under photoneural regulation, which involves an adrenergic and cAMP-dependent mechanism (*37*).

The distribution pattern of RORβ indicates that RORβ is most highly expressed in tissues involved in processing sensory information and in anatomical components implicated in the regulation of circadian rhythm. The latter is supported by observations showing fluctuations in RORβ2 mRNA expression with circadian changes and suggests that the RORβ2 promoter is controlled by the circadian clock (*35, 37*). Thus, RORβ2 may regulate genes encoding proteins involved in the regulation of the processing of sensory information and circadian rhythm. As discussed below, abnormalities in circadian behavior observed in ROR$\beta^{-/-}$ mice are in agreement with such a hypothesis.

C. RORγ

hRORγ was first cloned by PCR using poly(A)$^+$ RNA from human pancreas and two degenerate primers, the sequences of which were based on the two most highly conserved regions in the DBDs of the RAR and RXR receptors (*27*). The murine homolog of RORγ, also named TOR, was cloned by screening a mouse muscle (*28*) and a T cell cDNA library (*38*). Two different isoforms, referred to as RORγ1 and RORγ2 (also named RORγt), have been identified (*39*). The RORγ2 lacks an A/B domain and therefore is a truncated form of RORγ1; it

is derived by transcription from an alternative promoter (*40*). Northern blot analysis indicated that RORγ generates two mRNAs of different size, 2.4 kb and 3.5 kb. These different-size transcripts are derived from the use of two alternative polyadenylation signals (*28*).

RORγ1 has been identified in many tissues and is most highly expressed in the thymus, skeletal muscle, liver, mammary gland, and kidney. It is also highly expressed in brown fat tissue but not in white fat tissue, suggesting a possible role in the regulation of brown fat–specific genes (*27, 28, 38*). However, both RORγ and RORα have been shown to be induced during adipocyte differentiation in cultured 3T3-L1 and D1 preadipocytes, which function as *in vitro* models for white fat cell differentiation (*41*). The cytokines, TNF-α and TGF-β1, which inhibit adipocyte differentiation, also suppress the induction of RORα and -γ. What function RORγ has in fat cell differentiation awaits further study.

RORγ1 transcripts can be found in all tissues where RORγ is expressed, but RORγ2 transcripts are restricted to the thymus. In the thymus RORγ2 is most highly expressed in double-positive (DP) $CD4^+CD8^+$ thymocytes but not in mature, single-positive (SP) $CD4^+$ or $CD8^+$ thymocytes or in thymic epithelial cells (*39, 40, 42*). RORγ2 mRNA is also found in immature, double-negative (DN) $CD44^+CD25^-$ cells but not in other subpopulations of DN thymocytes. These observations indicate that RORγ2 expression is tightly controlled during thymopoiesis and suggest that RORγ2 regulates gene expression at discrete stages of T cell development. Expression of RORγ has also been observed in the murine thymocyte-like cell line S49, in a number of T cell lymphomas (including mouse EL-4 and YAC-1 cells), and human cutaneous T cell lymphoma HUT78 (*38*). RORγ is undetectable in spleen, bone marrow, natural killer (NK) cells, and B lymphocytes. No expression of RORγ mRNA was found in several B cell lymphomas and monocytic cell lines. In E14.5 embryos, RORγ has been found in regions where lymph nodes develop; particularly, $CD3^-CD4^+CD45^+$ IL-$7R\alpha^+$ lymph node precursor cells express high levels of RORγ mRNA (*43*). These findings suggest a role for RORγ in lymph node development. The latter is supported by the observed absence of lymph nodes in $ROR\gamma^{-/-}$ mice (*43, 44*).

D. Insect Homologs of ROR

DHR3 and MHR3 are genes identified in *Drosophila melanogaster* and *Manduca sexta*, respectively; these genes encode transcription factors structurally related to the nuclear hormone receptor family (*45–52*). Sequence comparison has indicated that these genes are most closely related to the ROR subfamily and may represent the insect homologs of ROR. In particular, their DBD and AF-2 regions show high homology with those of RORs. Recent studies have demonstrated that DHR3 is required for the prepupal–pupal transition and differentiation of adult structures during *Drosophila* metamorphosis (*45, 53*). Mutant DHR3 has been shown to cause defects in pattern formation of

the peripheral nervous system (*47*). These studies indicate that DHR3 plays an important role in the regulation of normal *Drosophila* development.

20-Hydroxyecdysone, a hormone that controls insect molting and metamorphosis, has been demonstrated to induce DHR3 and MHR3 expression (*53*). This induction is not immediate and requires protein synthesis. Although several ecdysone REs have been identified in the promoter of MHR3, only one of the three putative ecdysone REs has been found to bind a heterodimeric complex consisting of the ecdysone receptor EcR-B1 and the RXR homolog USP-1 (*51*). Future studies must determine whether this binding site has any functional role in the regulation of DHR3 and MHR3 by 20-hydroxyecdysone *in vivo* or whether this control occurs via an indirect mechanism.

III. Structure of ROR Proteins

The RORs have a domain structure very similar to that of other members of the nuclear receptor family and contain an amino-terminal domain, DBD, hinge domain, and LBD (*1, 25–28, 30, 38*). The different isoforms generated by each ROR gene differ only in their amino-terminal sequence (*30, 36, 39, 40*). A

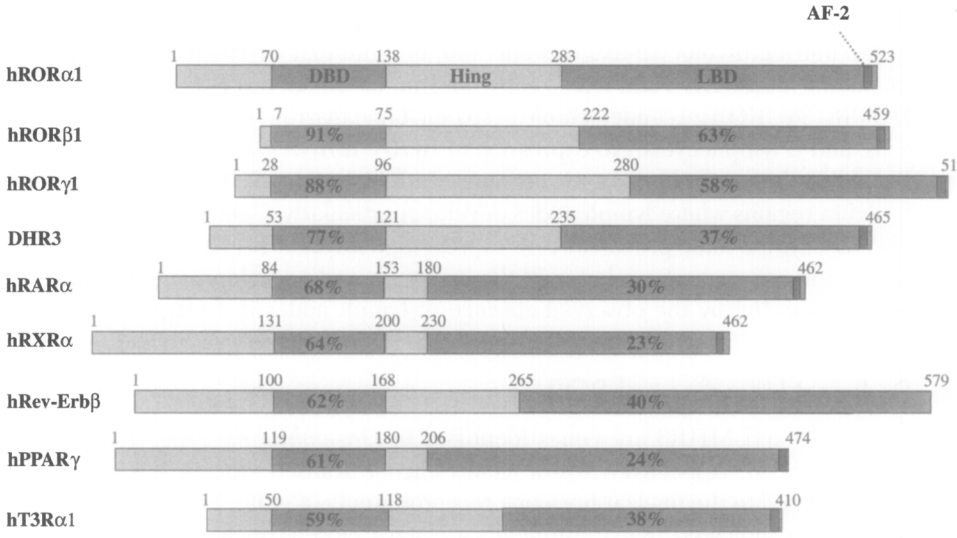

FIG. 1. Comparison of the modular structures of RORs with those of several other nuclear receptors. The percentages indicate the percent homology of the respective DBD or LBD with those of the RORα1 receptor. The AF2 regions are indicated by black boxes. DHR3, *Drosophila* homolog of RORs; RAR, retinoic acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator activated receptor; T3R, thyroid hormone receptor.

schematic comparison of the different domains in human RORα1, -β1, and -γ1 and those of several other nuclear receptors is shown in Fig. 1. RORγ1 exhibits a 54% and 51.5% identity with RORα1 and RORβ1, respectively, while RORβ1 and DHR3 are, respectively, 64.6% and 38.7% identical to RORα1. The DBD is the most highly conserved domain among RORs. The DBDs of RORβ and RORγ are, respectively, 91% and 88% identical to the DBD of RORα. The DBD of the *Drosophila* homolog DHR3 is 77% homologous to that of RORα. The DBD of the RAR receptor shares the next highest (68%) identity with

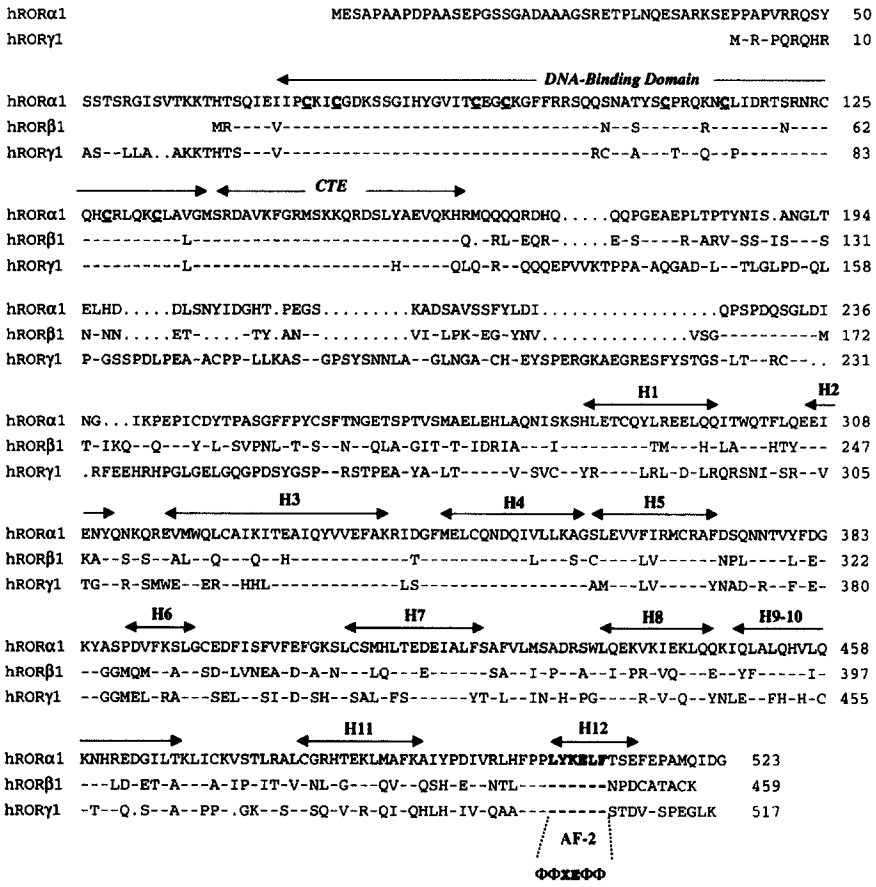

FIG. 2. Amino acid sequence comparison of human RORα1, -β1, and -γ1. Amino acids in RORβ1 and -γ1 that are identical to those in RORα1 are indicated by dashed lines; gaps are indicated by dotted lines. The DBDs and CTEs are most highly conserved among RORs. The cysteines that are part of the two zinc-finger motifs in the DBD are underlined and bold. The 12 helices (H1–12) of the LDB are indicated. H3-5 and H12 exhibit the highest degree of homology among RORs. H12 contains the AF-2 consensus motif ΦΦXEΦΦ.

those of RORs, while the DBDs of all other receptors exhibit less homology. The hinge domains of RORs exhibit little homology, while the LBDs of RORs are moderately conserved. The LBDs of RORβ and RORγ exhibit, respectively, a 63% and a 58% identity with the LBD of RORα. Among vertebrate receptors, the LBDs of RORs are most closely related to those of Rev-Erb and T3R receptors, exhibiting 35–40% identities among one another.

A comparison of the amino acid sequences of RORα1, RORβ1, and RORγ1 is shown in Fig. 2. In addition to the zinc-finger region of the DBD, the carboxyl-terminal extension (CTE) of the DBD is also highly conserved. The CTE has been shown to play a role in determining the affinity of RORα to RORE (9), as discussed below in more detail.

As has been demonstrated for other nuclear receptors (*11, 12*), the LBD of RORs contains 12 α-helical regions. Two regions in the LBD, one comprising helices 3–5 and the other helix 12, are particularly highly conserved among RORs. The helices 3–5 form the interaction surface for several coactivators and corepressors. The helix 12 region, consisting of the sequence PPLYKELF at the carboxyl terminus, is absolutely conserved among the three RORs and contains the consensus AF-2 motif $\Phi\Phi$XE$\Phi\Phi$(Φ represents a hydrophobic amino acid, and X is any amino acid) (*54*). The AF-2 in the *Drosophila* homolog DHR3 differs from that of ROR in only one amino acid. As will be discussed below, the AF-2 domain has a critical function in controlling the interaction of RORs with corepressors and coactivators, and hence the activity of RORs.

IV. Characterization of ROR Response Elements

The characteristics of the interactions of nuclear receptors with REs can vary substantially among receptors. A nuclear receptor can bind an RE as a monomer, as a homodimer, or as part of a heterodimer. Formation of heterodimeric complexes is usually in partnership with one of the retinoid X receptors (RXRs). Dimeric complexes can interact with direct, everted, or inverted (palindromic) repeats of the core motif AGGTCA spanned by 0–7 nucleotides (*3, 7*). Monomeric receptor binding occurs to REs containing variations of the single-core motif. To define the consensus sequence of hormone response elements that bind RORs, an electrophoretic mobility assay (EMSA)/PCR-based strategy was used that selects for oligonucleotides with the highest affinity for ROR from a pool of degenerate oligonucleotides. These studies revealed that RORs bind with highest affinity to DNA elements, referred to as ROREs, consisting of the core motif AGGTCA preceded by an AT-rich sequence (*26, 28, 30, 38, 55*). Variations in the A/T-rich half of the RORE can greatly influence the binding of ROR, indicating the importance of this sequence in determining the affinity and specificity of ROR binding.

Two-hybrid analysis and EMSA have shown that RORs bind to ROREs as monomers and do not form homodimers (*26, 28, 30, 55*). Although EMSA using a DR7 response element has demonstrated the formation of two ROR:nucleotide complexes representing the binding of either one or two RORs, the binding of the two ROR molecules appears to occur independently and does not involve dimerization of the two ROR proteins. A number of nuclear receptors have been reported to form heterodimeric complexes with RXRs; however, RORs have been found to be unable to heterodimerize with RXRs (*26, 30*).

Deletion mutation analysis has demonstrated that the two zinc fingers are not sufficient for ROR binding to RORE and that additional regions are required (*9*). To identify such regions, the effects of several amino- and carboxyl-terminal deletions on the binding of RORα1 to RORE were examined. Deletion of the amino terminus of RORα1 up to Ser_{35} (Fig. 2) had little effect on the binding of RORα1 to RORE; however, deletion of an additional 10 residues caused a dramatic reduction in binding, while deletion of another 10 amino acids did not further decrease binding. These results suggest that the region of RORα1 between Ser_{35} and Val_{45} is important for optimal binding. C-Terminal deletions up to Gln_{166} had little effect on the binding of RORα1; however, deletion up to Lys_{150} totally abolished binding. These results indicate that deletion of the LBD does not affect binding in a major way, suggesting that the LBD is not required for optimal binding. However, the CTE, the region flanking the C-terminal side of the DBD from Met_{138} to Gln_{166}, is critical for optimal RORα binding (*9*). The CTE is highly conserved among RORs (Fig. 2) as well as Rev-Erb receptors but shows little homology with CTEs of other receptors, including NGFI-B and SF-1, which likewise bind as a monomer to similar REs (*56–58*). Specific mutations within the CTE region totally abolished DNA binding of RORα, supporting its critical importance in ROR binding. Methylation interference studies suggested that the zinc fingers of RORα containing the P-box contact the major groove at the AGGTCA half of the RORE, while the CTE interacts with the adjacent minor groove at the 5′-A/T-rich half of the RORE (*9*).

Although all ROR receptors bind REs consisting of the core motif AGGTCA preceded by an AT-rich motif, different isoforms exhibit distinct affinities for different ROREs, as has been demonstrated for RORα1 and -α2 and RORβ1 and -β2 (*30, 36*). Since the amino terminus is the only difference in amino acid sequence between ROR isoforms, this region is likely involved in influencing the RORE binding specificity of RORs. This was corroborated by experiments comparing the binding specificities of RORα2 mutants carrying various deletions in the amino terminus. These results showed that such mutations greatly influenced the binding of this receptor to ROREs (*30*). In addition, experiments using hybrid receptors in which the amino terminus of the thyroid hormone receptor β (T3Rβ) was replaced by the amino terminus of either RORα1

or -α2 showed that the ROR amino-terminal domains impose DNA-binding specificity upon the heterologous nuclear receptor. The mechanism by which the amino terminus controls the binding affinity for ROREs has not yet been fully established. Although the amino terminus could make contacts with the AT-rich region itself and provide an additional DNA-binding site for the receptor, experimental evidence appears not to support this concept. Based on circular permutation and methylation interference analysis of ROR–RORE complexes, it was proposed that changes in the amino terminus alter the tertiary structure of the DBD and adjacent CTE, thereby affecting their contacts with DNA (59). The latter may explain the differences in affinity of ROR isoforms for different ROREs, but also provide a mechanism for differential regulation of target genes by ROR isoforms.

In the case of RORγ, the γ2 isoform is a truncated form of γ1 and has only three additional amino acids upstream from the DBD (*39, 40*). Both isoforms are able to bind the consensus RORγ-RE with high affinity and to enhance RORE-dependent transactivation to a similar degree (*60*). These results suggest that the amino terminus is not a requirement for binding and transactivation. However, these observations do not rule out a role for the amino terminus in finetuning the binding specificity of RORγ, as has been reported for RORα (*30*).

Some of the binding characteristics of RORs are shared with those of other nuclear receptors, such as Rev-Erbα and -β, SF-1, RTR, Nur77, and estrogen receptor–related receptors (ERRs) (*57, 58, 61, 62*). Therefore, these receptors could bind some of the same REs and compete with each other for binding. The type and extent of cross-talk between different receptor signaling pathways depends on whether the receptors are coexpressed in the same cell, the presence of their respective ligands and cofactors, and the affinities of the receptors for the same RE. The orphan receptors Rev-Erbα and -β have been reported to act as dominant-negative repressors of transcription and can bind to some of the same REs to which RORα and RORγ bind. Therefore, by competing for the same DNA-binding site, Rev-Erb can inhibit the transcriptional activation by ROR (*28, 41, 63–65*). In the case of N-Myc, the reverse has been reported. The repression of N-Myc by Rev-Erbβ can be abrogated by expression of RORα through a mechanism that involves competition between ROR and Rev-Erbα for the same RE (*65*).

A number of nuclear receptors bind as part of a heterodimer to REs consisting of a direct repeat (DR) spanned by 0–7 nucleotides. Depending on the specific sequence of these DRs, ROR has been found to be able to suppress the transcriptional activation mediated by some receptors by competing for binding to the same site. For example, the CRBPI gene contains a DR2 that is able to bind the RAR/RXR heterodimer. RORβ is able to bind this RE as well and competes with RAR/RXR for binding to this RE (*26*). Similar observations have been reported for RAREs and TREs (*38*).

V. Transcriptional Control by RORs

A. Ligand-Dependent or -Independent Activation?

Nuclear receptors can function as repressors as well as activators of transcription, and for many receptors these activities are controlled by ligands. Crystallographic studies with retinoid and PPAR receptors have demonstrated that ligand binding causes a change in the conformation of the receptor that results in the dissociation of a corepressor complex and the association of a coactivator complex (*11–13, 15, 16*). The coactivator complex induces through histone acetylation local changes in chromatin structure and mediates interaction of the receptor with the basal transcriptional machinery. Stimulation of RNA polymerase II activity then results in enhanced transcription of target genes. However, for certain receptors, such as the constitutive androstane receptor (CAR), androstanol binding acts in the reverse manner and results in repression of target gene expression (*66*).

With the discovery of nuclear orphan receptors, a number of questions have been raised about receptor activation by ligands. Do all nuclear receptors have ligands, or do certain receptors act as constitutive repressors or activators of transcription? Are certain receptors activated by mechanisms other than ligand binding, such as phosphorylation? Most, if not all, nuclear receptors appear to be phosphorylated. Alterations in phosphorylation can affect the receptor in a variety of ways, including modulation of their activity and protein stability. For example, phosphorylation of the PPARγ receptor at its amino terminus by a MAPK-activated signaling pathway has been shown to inhibit transcriptional activation by this receptor (*67, 68*). Mutation of a single tyrosine in the LBD of the estrogen receptor results in a constitutively active receptor (*69*), while phosphorylation of a serine residue in the hinge domain of SF-1 enhances the transactivation by this receptor (*70*). Phosphorylation sites in the glucocorticoid receptor have been reported to be involved in the control of its stability (*71*).

Like other nuclear receptors, RORs are likely phosphoproteins; however, the precise sites of phosphorylation have not yet been determined. RORα1 contains potential protein kinase C phosphorylation sites at Ser_{35} and Thr_{53} and a potential protein kinase A phosphorylation site at Ser_{49} (*9*). RORγ also contains several potential PKA and PKC phosphorylation sites. The AF-2 domains of RORs contain a Tyr residue that could be a target for phospho-Tyr kinases. As discussed below, mutation of this residue into Phe abrogates the interaction of RORγ with the steroid receptor coactivator-1 (SCR-1) and abolishes the transactivating activity of RORγ but has no effect on its interaction with the nuclear receptor corepressor (N-CoR) (*60, 72, 73*). These results indicate the importance of this residue in the activation function of RORγ. Although this Tyr may have only a structural role, its phosphorylation could control the

activation of ROR by inducing a conformational change in its LBD and promoting the association of coactivators, such as SRC-1. Future studies have to determine whether phosphorylation of this Tyr plays any role in regulating the activity of RORs.

RORs are considered orphan receptors because it is not known whether their activity is regulated by ligands. Reporter gene assays, in which an ROR expression vector and an RORE-dependent reporter gene plasmid are cotransfected into mammalian cells, have demonstrated that RORs are potent activators of transcription in many cell types (*26, 28, 38, 55*). In general, it appears that one RORE is insufficient to induce ROR-mediated transactivation and that two or more ROREs are required to obtain optimal transcriptional activation. The absence of fetal calf serum from the medium does not influence transactivation by RORs, suggesting that potential ligands in serum are not required for ROR-mediated transactivation (*38*). Interestingly, transcriptional activation by both RORα and RORβ has been reported to be cell type–dependent. RORβ can increase RORE-dependent transcription in neuronal cells but not in nonneuronal cells (*55*), while RORα-mediated transcriptional activation has been observed in human choriocarcinoma JEG-3 cells but not in human kidney 293T cells (*74*). This apparent cell type–specific transactivation by RORs could be due to the cell type–specific expression or activation of one or more coactivators. Alternatively, the activation of ROR itself could be cell type–specific and depend on the cell type–specific synthesis of a ligand or phosphorylation of ROR or cofactors by a cell type–specific kinase.

Since RORβ is highly expressed in the pineal gland, the principal source for melatonin, it has been hypothesized that melatonin could be a ligand for RORs. Initial studies reported that melatonin was able to bind to RORβ and to enhance the transcriptional activation by RORβ (*34, 75*). However, subsequent studies by several laboratories were unable to demonstrate binding and activation by melatonin (*55, 76*) (A. M. Jetten, unpublished results), indicating that melatonin does not function as a ligand for RORs. Several thiazolidine derivatives, including CGP52608 which has potent antiarthritic activity, have been reported to enhance specifically the transactivation by RORα and RORβ (*77, 78*). However, further analysis is needed to confirm this agent as a true synthetic ligand for RORs. Therefore, the question remains: Do RORs act as constitutively active receptors or are their activities controlled by a ligand-dependent or -independent mechanism?

B. Interaction of ROR with Corepressors and Coactivators

Transcriptional activation by nuclear receptors is mediated through interaction with multiprotein coactivator complexes that consist of histone acetylases, coactivators, and other cofactors (*13, 15, 16*). Recently, an increasing number

of cofactors have been identified, including SRC-1, glucocorticoid receptor–interacting protein-1 (GRIP-1, also known as TIF-2 or N-CoA-2), receptor-interacting protein 140 (RIP-140), T3R-interacting proteins (TRIPs), T3R-associated proteins (TRAPs), and cAMP response element–binding protein (CBP) (*13, 15, 16*). Some of these cofactors bind to a limited number of nuclear receptors whereas others exhibit a low specificity.

Mammalian two-hybrid analysis has demonstrated that RORα can interact with coactivators TRIP-1, transcription intermediary protein-1 (TIF-1), TRIP-230, peroxisome proliferator–binding protein (PBP; also named hTRAP220), and GRIP-1 (*79*). RORγ can interact with several coactivators, including SRC-1 and CBP (*60*). Pulldown analyses have demonstrated that these coactivators physically interact with RORs. In addition, SRC-1, GRIP-1, and CBP are able to enhance ROR-mediated transcription, suggesting a physiological role for these coactivators in the induction of gene expression by RORs (Fig. 3). GRIP-1 and

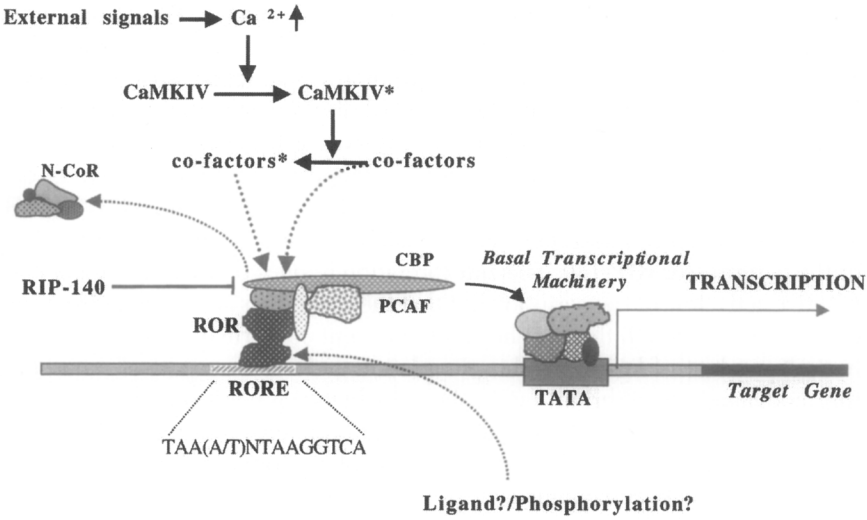

FIG. 3. Model of transcriptional activation by RORs and the potential role of CAMKIV. RORs bind as a monomer to ROR-response elements (ROREs) consisting of the consensus core motif AGGTCA preceded by an AT-rich sequence. In the transcriptionally inactive form, RORs interact with a corepressor complex and repress transcription. Ligand binding and/or phosphorylation induce(s) changes in the conformation of ROR, causing dissociation of the corepressor complex and association of a coactivator complex. The corepressor RIP-140 may compete with coactivators for binding to ROR, thereby inhibiting transactivation. Signaling pathways that increase Ca^{2+} result in the activation of CaMKIV and the subsequent phosphorylation of one or more nuclear cofactors. Phosphorylation of such cofactors may increase its affinity for ROR, promote the assembly of specific coactivator complexes, and induce ROR-mediated transactivation. An asterisk indicates activated or phosphorylated.

CBP exhibit intrinsic histone acetylase activity that leads to acetylation of nucleosomal histones, opening of the chromatin structure, and subsequently enhanced transcription.

RORγ also interacts with RIP-140 (*60*) which has been reported to function as a corepressor as well as a coactivator (*80, 81*). RIP-140 was shown to suppress RORE-dependent transactivation by RORγ (*60*). Similarly, PBP decreased rather than increased the transactivation by RORα (*79*). These observations indicate that RIP-140 and possibly PBP function as repressors of ROR-mediated transcriptional activation, likely by competing with coactivators for ROR binding (Figs. 3 and 4).

Although RORγ is a very effective inducer of transcription, it is also able to interact with the corepressor N-CoR in both two-hybrid and pulldown analyses (*60*). RORγ is unable to interact with the corepressor SMRT (silencing mediator for retinoic acid and thyroid hormone receptor). Thus, RORγ can interact with both the corepressor N-CoR and the coactivator SRC-1. Studies with several other nuclear receptors have demonstrated that, upon ligand binding, the LBD of the receptor undergoes a conformational change. The apo-receptor is usually transcriptionally inactive and permits binding of corepressors while the conformation of the holo-receptor promotes interaction with coactivators (*11–13, 15, 16*). It appears unlikely that ROR displays only one conformational state that enables it to interact with corepressors as well as coactivators. It is more likely that ROR can assume two or more different conformations (Fig. 4); one conformation allows interaction of RORγ with the corepressors, such as N-CoR, while another conformation permits association with coactivators, such as SRC-1, or the corepressor RIP-140. This interpretation implies that RORs are not constitutive activators of transcription but that their activities are regulated by some mechanism. Although the shift between different conformations of ROR could be independent of ligand or phosphorylation and occur as part of a constant thermodynamic equilibrium (as in the shift between conformation I and III; Fig. 4), it appears more likely that the transition between different conformations is controlled by ligand binding or through phosphorylation by protein kinase (as in the shift between conformations I and II; Fig. 4).

A number of different regions in the nuclear receptor have been implicated in the interactions of receptors with corepressors and coactivators. Some of these regions serve as an interaction surface, while others control binding through conformational changes in the LBD. Deletion and point mutation analyses were carried out to identify the regions important in the interaction of RORs with corepressors and coactivators. Amino-terminal deletion up to Q_{221} of RORγ1 largely abolishes the interaction with N-CoR but has little effect on the binding of SRC-1 and RIP-140 (*60*). These results suggest that the amino-terminal region of the hinge domain is important for the binding of N-CoR. It is, however, not required for the interaction of ROR with SCR-1 or RIP-140. The hinge domain has also been implicated in the binding of N-CoR to other receptors,

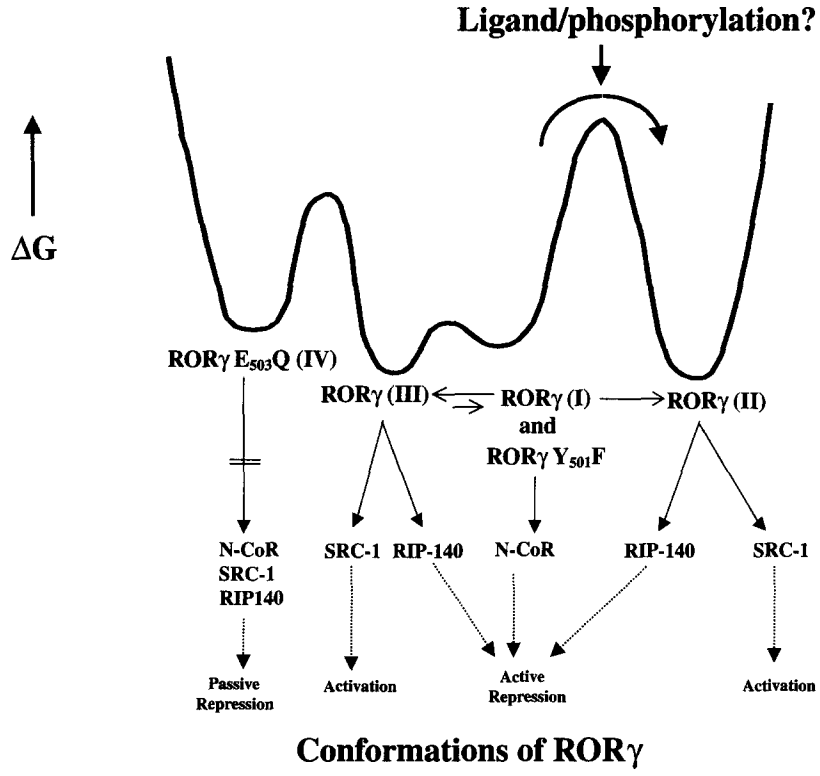

FIG. 4. The transcriptional activity of RORs is dependent on different conformations. ROR in conformation I can interact with the corepressor N-CoR and acts as an active repressor, while conformations II and III promote interaction with SRC-1 and RIP-140, resulting in activation or repression of transcription, respectively. Although the shift between two conformations (e.g., I and III) may not require ligand binding or phosphorylation of ROR, it appears more likely that the transition is regulated by ligand binding and/or phosphorylation by a specific protein kinase (as the shift between I and II). The point mutation $Y_{501}F$ in hRORγ1 abolishes binding of SRC-1 and RIP-140 but does not affect its interaction with N-CoR. This mutation may retain RORγ in a conformation similar to I and make RORγ behave as a constitutive repressor. The mutation $E_{503}Q$ abolishes binding of hRORγ to SRC-1, RIP140, and N-CoR and may represent another conformation (IV) of RORγ1 which functions as a passive repressor (*16*).

but the regions within the hinge domain required for this interaction vary among receptors and do not exhibit any sequence similarities (*72*). It appears that these regions in the hinge domain are structurally important instead of providing an interaction surface for N-CoR.

Helix 12 and helices 3–5 in the LBD of nuclear receptors have been reported to be critical elements in the binding of coactivators and corepressors

(*15, 16, 82*). Helices 3–5 are part of the interaction surface for the LXXLL motif in coactivators, such as SRC-1 and CBP, as well as for certain corepressors (*83–85*). This region is moderately conserved among ROR receptors (see Fig. 2). As expected, deletion of this region totally abolishes the ability of ROR to bind the coactivators SRC-1, CBP, and GRIP-1, and the corepressor RIP-140 (*60, 79*). In addition, RORα(V_{335}R) containing a point mutation in helix 3 no longer interacts with either GRIP-1 or PBP.

Helix 12 region constitutes the carboxyl-terminal end of RORs (Fig. 2). The amino acid sequence of helix 12 contains the nuclear receptor AF-2 consensus sequence ΦΦXE/DΦΦ (*54*). This region has been demonstrated to play a critical role in controlling the binding of coactivators and hence the activity of the receptor (*82, 86, 87*). The role that helix 12 plays in the interaction of nuclear receptors with corepressors is somewhat different for each receptor. Deletion of the AF-2 region in RORγ or RORα completely abolishes its interaction with SRC-1, CBP, GRIP-1, PBP, N-CoR, and RIP-140 (*60, 79*). The AF-2 point mutation Y_{501}F does abolish the binding of RORγ1 to SRC-1 and RIP-140 but does not affect the interaction with N-CoR (*60*). This mutation may retain RORγ in an inactive conformation (similar to conformation I in Fig. 4), making it behave as a constitutive repressor. Whether Tyr_{501} has only a structural role or whether its potential phosphorylation can modulate the conformation and activity of RORs has yet to be established. The AF-2 mutation E_{503}Q abolishes the binding of RORγ to SRC-1, RIP-140, and N-CoR, indicating that this mutation induces a change in conformation of the LBD (as in conformation IV in Fig. 4) that does not allow interaction with any of these three proteins. The fact that different mutations affect the binding of SRC-1 and N-CoR differently suggests that each mutation induces a different conformational change in the DBD of RORγ (Fig. 4).

Recently, using RORβ as a bait in yeast two-hybrid screening, a novel protein referred to as neuronal interacting factor X1 (NIX1) was identified (*88*). In addition to binding RORβ, NIX1 was also able to interact with ligand-bound RAR and T3R but not with RXR or several steroid hormone receptors. NIX1 is exclusively expressed in brain with significant expression in the dentate gyrus of the hypocampus and in the thalamus, hypothalamus, and brainstem nuclei. NIX1 is a 27-kD nuclear protein that contains two LXXLL motifs. These motifs are found in many coactivators and are critical elements in receptor–cofactor interactions. The AF2 of RORβ is required for NIX1 binding, and only one of the LXXLL motifs in NIX1 is necessary for binding RORβ. No intrinsic transcriptional activity is associated with NIX1, and like RIP-140, it inhibits transactivation by RORβ, possibly by competing with coactivators for receptor binding.

Two-hybrid analysis identified the nucleoside diphosphate kinase NM23 and the coactivator TRIP-1 as proteins interacting with RORβ (*89*). NM23 has been reported to play a role in organogenesis and differentiation, and its expression

is inversely related to metastasis. Pulldown analysis confirmed interactions of NM23 with RORα or -β. However, whether these interactions have any physiological significance has yet to be established.

C. Regulation of ROR-Dependent Transactivation by CaMKIV

CaMKIV is a multifunctional Ser/Thr protein kinase that can phosphorylate a variety of substrates (*90*). CaMKIV is expressed in several tissues, including brain, T lymphocytes, and testis, where it is found in spermatogonia and spermatids (*90, 91*). CaMKIV is rapidly activated upon elevation of the intracellular Ca^{2+} concentration and is predominantly localized to the nucleus. Its nuclear localization suggested a possible role for CaMKIV in the regulation of transcription. This was supported by reports showing that several transcription factors, including cAMP response element–binding protein (CREB), activating transcription factor-1 (ATF-1), and serum response factor (SRF), are targets for CaMKIV phosphorylation (*92, 93*).

Recent studies have shown that CaMKIV can also enhance transcriptional activation mediated by members of the ROR family (*94*). Cotransfection of expression vectors encoding RORα and a Ca^{2+}/calmodulin-independent form of CaMKIV enhanced RORE-dependent transcriptional activation of a reporter gene 20–30-fold. Cotransfection of a catalytically inactive CaMKIV had no effect (*94*). Stimulation of ROR-mediated transactivation was also observed in epidermal HaCaT cells after activation of endogenous CaMKIV by the Ca^{2+}-ionophore ionomycin. CaMKIV was able to enhance not only transcriptional activation mediated by RORα1, but also that by RORα2 and RORγ and, to a lesser extent, that by COUP-TF1. CaMKIV did not increase T3Rα- or ER-mediated transactivation, indicating that this type of activation is limited to a distinct group of nuclear receptors. Stimulation of ROR-mediated transactivation was also observed with CaMKI but not with CaMKII. Deletion studies demonstrated that the LBD of RORα is required for the CaMKIV-induced activation. Although RORα contains two putative CaMKIV phosphorylation sites at the amino terminus, mutation analysis indicated that these sites are not involved in CaMKIV-induced transactivation. In addition, CaMKIV was unable to phosphorylate *in vitro* transcribed RORα (*94*). These observations suggest that the increase in ROR-mediated transactivation by CaMKIV may involve phosphorylation of other proteins. Since transactivation by RORs is mediated through interactions with other nuclear proteins, such cofactors may be putative targets for CaMKIV phosphorylation. Alternatively, CaMKIV-stimulated transactivation could result from modification of a biosynthetic enzyme involved in the production of ROR ligands or activation of another kinase.

As discussed above, RORs can interact with several nuclear cofactors, including SRC-1, GRIP-1, CBP, and p300 (*60, 79, 89*). Any of these cofactors could potentially be involved in the CaMKIV-induced transactivation by RORs. These coactivators interact with the LBD of nuclear receptors through their signature LXXLL motifs (*13*). Two-hybrid analyses examining the interaction of VP16-RORα(LBD) with a series of Gal4(DBD)-peptides containing various LXXLL motifs showed that several peptides containing the consensus HVXXHPLLΦXLL are able to bind RORα (*94*). Constitutively active CaMKIV dramatically enhances transactivation in this two-hybrid system. These peptides are also able to inhibit CaMKIV-stimulated, RORE-dependent transactivation by RORα1 and RORγ. The sequence HVXXHPLLΦXLL has not yet been identified in any known cofactor, suggesting that a novel, as yet unidentified, cofactor may mediate ROR-dependent transactivation. Since CaMKIV is a Ca^{2+}-dependent kinase, one could hypothesize that the transcriptional activation by RORs may be modulated by Ca^{2+} influx through the activation of CaMKIV. Therefore, signaling pathways that induce Ca^{2+} influx should be able to dramatically enhance ROR-dependent transactivation in cells that express both RORs and CaMKIV (*94*). Figure 3 shows a putative model of the mechanism of ROR-mediated transcriptional activation and the potential role of CAMKIV.

It is interesting to note that CaMKIV is expressed in several tissues, including the cerebellum, retina, and thymus, where RORs control important functions (*27, 32, 36, 39, 90*). In addition, CaMKIV$^{-/-}$ mice exhibit several phenotypic changes similar to those observed in ROR$\alpha^{-/-}$ mice (*94*). However, in contrast to ROR-knockout mice, spermatogenesis is greatly affected in CaMKIV$^{-/-}$ mice and the mice are infertile (*95*), suggesting that this phenotype involves alterations in signaling pathways other than RORs. Thymocytes from mice expressing a catalytically inactive CAMKIV undergo rapid cell death when placed in culture, as do thymocytes from ROR$\gamma^{-/-}$ mice (*43, 44, 91*). These observations further support a possible link between CaMKIV and ROR-mediated transcriptional activation, at least in the regulation of certain biological processes.

VI. Genomic Structure and Chromosomal Localization

The genomic structure of the RORγ gene was determined from a P1 vector clone containing the entire mouse RORγ gene (*96*). A schematic representation of the RORγ genomic structure is shown in Fig. 5. The mouse RORγ gene spans more than 21 kb and consists of 12 exons separated by 11 introns. As mentioned above, the RORγ gene generates two isoforms that differ in their amino termini. The amino terminus of RORγ1 is encoded by two exons, 1a and 2, while that of

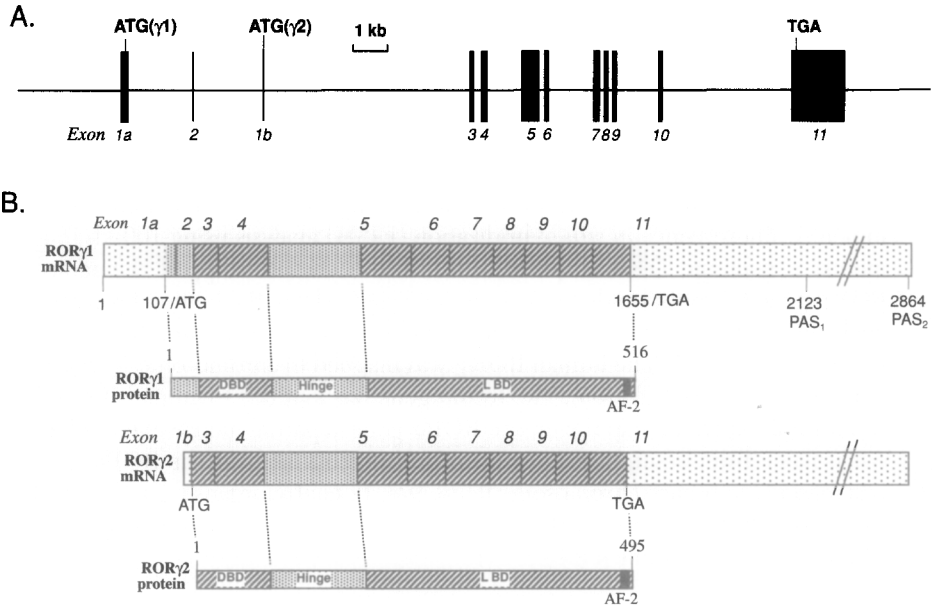

FIG. 5. (A) Schematic presentation of the genomic structure of the mouse RORγ gene. The RORγ gene consists of 12 exons (black boxes). The start and stop codons are indicated. (B) Comparison of the structure of RORγ1 and RORγ2 mRNA and protein. Sparse stippling indicates 5′- or 3′-UTR; diagonal stripes indicate DBD or LBD; dense stippling indicates amino terminus or hinge domain; black boxes indicate AF2 regions. The regions of the RORγ mRNAs corresponding to the various exons are indicated. Through the usage of alternative promoters, the RORγ gene generates two isoforms, RORγ1 and RORγ2. RORγ2 is identical to RORγ1 except that it lacks the amino-terminal domain of RORγ1. Exons 1a and 2 encode the 5′-UTR and amino terminus of RORγ1, while exon 1b encodes the 5′-UTR and three amino acids at the amino terminus of RORγ2. The RORγ gene generates several transcripts, 2.1 kb and 2.8 kb in size, by the usage of different promoters and alternative polyadenylation signals PAS_1 and PAS_2.

RORγ2 is encoded by a single exon, 1b (*39, 40, 96*). The positions of these exons are shown inFig. 5. Based on the genomic structure and the different cell type–specific patterns of expression exhibited by the two RORγ isoforms, one can conclude that these isoforms are regulated by different promoters. The DBD of RORγ, spanning the region from Cys_{31} to Cys_{91}, is contained within exons 3 and 4. Exon 5 encodes the hinge domain, while the remaining exons encode the entire LBD. The sites of several intron/exon junctions in RORγ are conserved with those in other nuclear receptors. The location of the second intron (at Ser_{24} in RORγ1) is shared with an equivalent splice site in the RORα gene. Intron 3 is located between the exons encoding the two zinc fingers of RORγ, at Lys_{54}. The location of this intron is identical to that of equivalent introns

in thyroid hormone and retinoid receptors but differs from those in steroid hormone receptors. The position of intron 4 at the C terminus of the DBD of RORγ (Ala_{100} in RORγ1) is highly conserved among nuclear receptors. Based on the locations of these splice sites, the receptors have been divided into several evolutionarily divergent subgroups. In this respect, RORs fit into the T3R/RAR subgroup.

The chromosomal localizations of mouse and human RORγ were determined by fluorescence *in situ* hybridization (FISH) analysis using 100-kb fragments of genomic DNA as probes (*96*). These studies mapped the mouse RORγ gene to a position that is 54% of the distance from the heterochromatic–euchromatic boundary to the telomere of chromosome 3, an area that corresponds to 3F2.1–2.2. The human RORγ was mapped to chromosome 1, an area that corresponds to 1q21 (*96*). The RORα gene was mapped to human chromosome 15q21–q22. To map the mouse RORα gene, a partial mouse cDNA clone was isolated from brain. Using interspecific backcross analysis, the RORα gene was mapped to mouse chromosome 9 (*33, 97*), 12 centimorgans from the thy-1 locus (*98*). RORβ was mapped to human chromosome 9q22, a region syntenic with mouse chromosome 4 (*99*).

VII. Targeted Knockouts of RORs

A. Phenotype of RORα$^{-/-}$ Mice

Disruption of the RORα gene has been linked to the phenotype observed in homozygous *staggerer* (*sg/sg*) mice (*31, 33*). This natural mutant mouse strain was first described in 1962 and the affected allele mapped to chromosome 9 where RORα also resides (*31, 33, 100, 101*). *Sg/sg* mice show tremor, body imbalance, small body size, and die shortly after weaning. These mice exhibit severe cerebellar ataxia due to a defect in Purkinje cell development. Defective development of the thymus and immunological abnormalities have also been reported in *sg/sg* mice (*102, 103*). Positional cloning using genetic and physical mapping revealed a 6.5-kb deletion in the genomic sequence of the RORα gene (*31, 33*) that results in the deletion of an exon encoding the amino-terminal part of the ligand-binding domain. This deletion also causes a shift in the reading frame at amino acid 273 of RORα1 and creates a premature stop codon 27 amino acids further. Such a deletion results in a truncated RORα that retains the DNA-binding activity of RORα but lacks the ligand-binding domain.

Mice lacking a functional RORα gene have also been generated by targeted disruption using a knockout vector in which the β-galactosidase (β-gal) or neomycin phosphotransferase (neo) gene replaced the second zinc finger of the DBD of RORα (*32, 104*). RORα$^{-/-}$ mice exhibit a phenotype very

similar to that of *sg/sg* mice (*32, 33*). As in *sg/sg* mice, ROR$\alpha^{-/-}$ mice have an abnormal body balance and die a month after birth. Their motor coordination is reduced, as indicated by increased stumbling frequency. Tests to determine muscle strength and equilibrium showed that these capabilities are significantly reduced. The morphological and electrophysiological characteristics of the cerebella from ROR$\alpha^{-/-}$ mice are indistinguishable from those of *sg/sg* mice. Subsequent studies have shown that the cerebellar cortex in ROR$\alpha^{-/-}$ mice is grossly underdeveloped. The granular layer is almost nonexistent and depleted of granule cells, while the Purkinje cells are immature and reduced in number (*32, 104*). Expression of calbindin and GAD67 mRNAs is unaffected in Purkinje cells from *sg/sg* mice, in agreement with the hypothesis that the *sg/sg* defect occurs in developing Purkinje cells after the initiation of differentiation (*105*). Although in heterozygous mice the morphology of the cerebellar cortex appears normal, a significant loss of cerebellar neurons occurs during aging. The onset of Purkinje cell loss occurs earlier in males than in females (*106*).

The thyroid hormone (T3) also plays a key role in cerebellar development. Hypothyroid rodents exhibit abnormal Purkinje cell neurogenesis similar to that seen in *sg/sg* mice (*107*). Since both RORα and T3R are expressed in these cells, the question has been raised as to whether there is any link between the mechanism by which these two receptor signaling pathways affect Purkinje cell neurogenesis and whether RORα acts upstream of the T3R receptor or the reverse. Interestingly, the response of Purkinje cells to T3 is blocked in *sg/sg* mice. The Purkinje cell protein-2 (pcp-2) gene has been identified as a putative target for regulation by T3R (*108*) and RORα (*109*). Expression of this protein is undetectable in *sg/sg* mice despite the presence of T3Rβ. One study has concluded that the effect of RORα on cerebellar development may be mediated through an influence on the T3 signaling pathway (*33*). A different study has shown that T3 can alter the timing of RORα expression during development and may, as a consequence, influence Purkinje cell neurogenesis (*110*). Another consideration is that a subset of TREs may serve as response elements for both RORα and T3R. Changes in the level of expression of either receptor may affect the competition between T3R and RORα for such binding sites and alter the transcription of specific genes. Future studies are needed to provide further insight into the precise mechanisms underlying the interactions between these two receptor-signaling pathways.

Although high levels of RORα are normally expressed in the suprabasal layers of the epidermis and in hair follicles, no changes in the epidermis of ROR$\alpha^{-/-}$ mice were observed (*32*). However, these mice develop a significantly less dense fur that grows back much more slowly after shaving. RORα is also expressed in testis; however, spermatogenesis in ROR$\alpha^{-/-}$ mice appears normal and the animals are fertile.

Recently, RORα has also been implicated in the control of bone metabolism (*111*). Bone is a metabolically highly active tissue in which homeostasis is maintained through a balance between the activities of osteoblasts and osteoclasts. RORα is expressed in human mesenchymal stem cells in bone marrow, and its expression is increased when these stem cells undergo osteogenic differentiation. A functional role for RORα in bone metabolism has been indicated by studies showing that *sg/sg* mice have thin long bones that are osteopenic (*111*). The total bone mineral content in the tibia was found to be significantly diminished in *sg/sg* mice compared to *sg/+* and *wt* mice. These results suggest a positive role for RORα in the regulation of bone metabolism and bone homeostasis. Osteoblasts produce a number of proteins, including collagen I, bone sialoprotein, osteopontin, and osteocalcin, important in the formation of the bone extracellular matrix and mineralization. An RORE has been identified in the promoter of the bone sialoprotein gene, and RORα has been shown to enhance transcription through this promoter in rat osteosarcoma ROS 17.2,8 cells (*111*). In contrast, RORα inhibits the activation of the osteocalcin promoter by vitamin D, suggesting potential cross-talk between these two receptor-signaling pathways.

The RORα gene also appears to influence the susceptibility to atherosclerosis (*112*). *Sg/sg* mice put on a 9-week high-fat diet developed many atherosclerotic lesions in the small and large coronary arteries and displayed a profound hypoalphalipoproteinemia. The latter was associated with decreased plasma levels of the HDL proteins, apolipoprotein AI and AII (apoA-I and A-II). The reduction in apoA-I levels was due to a decreased expression of the apoA-I gene in the intestine but not in liver. In this regard, it is interesting to note that RORα1 was shown to activate apoA-I transcription in intestinal Caco-2 cells (*113*). This activation might be mediated by the RORα-response element present in the promoter region of the apoA-I gene. These results suggest that apoA-I may be a potential target gene for RORα.

The *sg/sg* mutation also causes developmental and regulatory changes in the immune system (*101*). The development of the thymus is delayed; the spleen is undersized and the lymph nodes are enlarged. This immune phenotype is different from that observed in ROR$\gamma^{-/-}$ mice (*43, 44*). The formation of helper T cells appears normal, but *sg/sg* mice are deficient in the generation of suppressor cells (*103*). Splenocytes from *sg/sg* mice treated *in vitro* with lipopolysaccharide (LPS) show dramatically higher levels of induction of interleukin (IL)-1α, IL-1β, and TNFα than do those from wild-type mice treated with LPS (*102*). Similarly, treatment of *sg/sg* intraperitoneal macrophages with LPS or *N*-acetylmuramyl-L-alanyl-D-isoglutamine increases IL-1α mRNA and IL-1β protein to levels 5–10-fold higher than those attained in macrophages from wild-type mice, demonstrating that these agents induce a hyperexcitable state in macrophages from *sg/sg* mice.

B. Phenotype of ROR$\beta^{-/-}$ Mice

To investigate the biological role of RORβ, Andre and co-workers (*99*) disrupted the RORβ gene using a targeting vector in which the second zinc finger of RORβ was replaced by the β-gal gene. The pattern of β-gal activity generated in the ROR$\beta^{-/-}$ mice correlates well with the expression of RORβ. In agreement with *in situ* hybridization analysis (*35*), β-gal activity matches RORβ expression in the retina, pineal gland, spinal cord, and several areas in the brain. ROR$\beta^{-/-}$ mice do not show gross anatomical changes in the pineal gland, brain, or spinal cord of adult mice, suggesting that these tissues undergo normal development. Young ROR$\beta^{-/-}$ mice are undersized and initially manifest diminished muscular strength and ataxic movements. Later in adulthood they display a characteristic "duck-like" gait. The biological defect underlying this gait abnormality may be due to an impaired integration of sensory input information (*99*). The phenotype of the ROR$\beta^{-/-}$ mice resembles that of the extinct, spontaneous mouse strain *vacillans*. ROR$\beta^{-/-}$ mice are generally fertile, except that males do not sexually reproduce during the first 6 months. β-Gal activity is found in the epithelial cell lining of the epididymis and vas deferens, while testis and prostate are negative. No difference in this expression is observed between young and old ROR$\beta^{-/-}$ mice that could explain infertility at early age. Histological analysis of the eyes of adult ROR$\beta^{-/-}$ mice showed that the retina is greatly malformed. The retina is disorganized and seems to be collapsed. Shortly after birth, the developing retina of ROR$\beta^{-/-}$ mice is not very different from that of *wt* mice; however, several weeks later, the retina appears to exhibit defects in cellular differentiation and manifests degenerative cell loss (*99*). Adult mice are therefore blind, and tests to analyze the visual capabilities demonstrated that ROR$\beta^{-/-}$ mice do not have any visual activity. Although the ROR$\beta^{-/-}$ mice exhibit a very different circadian behavior, the system of nonvisual photoreceptors in the retina that mediates light-induced circadian responses was not impaired. Under constant darkness the free-running circadian period is lengthened by about 0.4 h. Mutations in several other proteins have been reported to increase the free-running period, including the prion protein and the transcription factor CLOCK, a member of the helix–loop–helix PAS family (*114, 115*). Whether RORβ acts upstream or downstream from these proteins has yet to be established. It has been suggested that RORβ might regulate the transcription of effectors of circadian rhythm, such as melatonin. Circumstantial evidence for this is provided by observations showing that RORβ is expressed in the pineal gland and in photoreceptors, the two principal producers of melatonin. In addition, the level of RORβ correlates with melatonin biosynthesis. For example, the onset of the rhythmic expression of RORβ in retina and pineal gland coincides with the induction of melatonin synthesis. Although melatonin could potentially act as a ligand for RORβ, several laboratories have demonstrated that this is not the case (*55, 76*).

Future studies are needed to determine the precise molecular mechanisms by which RORβ controls circadian rhythm.

C. Phenotype of ROR$\gamma^{-/-}$ Mice

1. Role for RORγ in Lymph Node Organogenesis

Two different laboratories have reported on the disruption of the RORγ gene in mice (*43, 44*). The general appearance of ROR$\gamma^{-/-}$ mice is normal and the mice are healthy during early stages of life. Their reproductive capacity is not compromised. Necropsy studies have revealed that ROR$\gamma^{-/-}$ mice lack all lymph nodes. Peripheral (e.g., popliteal, inguinal, cervical), paraaortic, and mesenteric lymph nodes as well as Peyer's patches are absent, indicating that lymph node development is arrested. In contrast, lymphatic vessels appear to be normal. These observations suggest that RORγ plays a critical role in lymph node organogenesis.

Recent studies have demonstrated the importance of several proteins in the regulation of lymph node development and include members of the tumor necrosis factor (TNF) family, their receptors, and the transcription factor Id2. Like ROR$\gamma^{-/-}$ mice, mice deficient in lymphotoxin (LT) α or LT receptor (LTR) β usually lack all lymph nodes and Peyer's patches and, in contrast to ROR$\gamma^{-/-}$ mice, have a disorganized spleen that lacks germinal centers (*116–119*). LT$\beta^{-/-}$ mice lack most lymph nodes but retain mesenteric and cervical lymph nodes (*120*). RANK is a member of the TNF receptor family; RANK$^{-/-}$ mice lack all lymph nodes except mucosal-associated lymph nodes and also display a deficiency in B lymphocytes and osteoclast differentiation (*121*). Alymphoplasia (aly) mice, which carry a point mutation in the NF-$\kappa\beta$–inducing kinase (NIK) gene, are characterized by the systemic absence of lymph nodes and Peyer's patches, disorganized splenic and thymic architectures, and immunodeficiency (*122, 123*). These observations indicate that lymph node organogenesis is complex and that formation of different lymph nodes involves control by different signaling pathways. Whether there is any link between expression of RORγ and the LT and LTR signaling pathways has yet to be investigated.

Mice disrupted in the Id2 gene, which encodes a transcriptional factor of the basic helix–loop–helix family, lack lymph nodes and Peyer's patches (*124*). In contrast to ROR$\gamma^{-/-}$ mice, Id2$^{-/-}$ mice also lack natural killer cells, but do not appear to exhibit any abnormal thymic or splenic phenotype. Recent studies have indicated that CD3$^-$CD4$^+$CD45$^+$ IL-7Rα^+ progenitor cells are important in the development of secondary lymphoid organs and NK cells (*125*). These early precursor cells express both RORγ and Id2 and are absent in ROR$\gamma^{-/-}$ and Id2$^{-/-}$ mice, which suggests that both of these transcription factors are essential for differentiation and/or survival of these progenitor cells (*43, 124*). Whethe the RORγ- and Id2-signaling pathways are engaged

in any cross-talk or whether RORγ acts down- or upstream of Id2 are intriguing questions that await further study.

2. Role for RORγ in Thymopoiesis and Apoptosis

In addition to the absence of lymph nodes, ROR$\gamma^{-/-}$ mice exhibit several changes in thymopoiesis (*43, 44*). At 2–3 months of age, the thymus is relatively smaller and the total number of T lymphocytes is reduced by 75%. The number of peripheral blood T lymphocytes in ROR$\gamma^{-/-}$ mice is about one-sixth that in wild-type mice, whereas the number of B lymphocytes does not change. Although the spleen contains almost 3 times more lymphocytes, splenic architecture is normal. The number of B lymphocytes in spleen is increased relative to that of T lymphocytes.

T lymphocyte maturation in the thymus is a well-defined, multistep process that involves proliferation, differentiation, apoptosis, selection, and commitment to different lineages (*126–133*). A schematic presentation of thymopoiesis is shown in Fig. 6. Early during thymopoiesis, the immature $CD4^-CD8^-$ double negative (DN) thymocytes, which represent a minority (3–5%) in the adult thymus, undergo a series of changes. The thymic lymphoid $CD44^+CD25^-$ progenitors differentiate via two intermediate stages, $CD44^+CD25^+$ and $CD44^-CD25^+$, into $CD44^-$ $CD25^-$ pre-T cells. During this differentiation, the T cell receptor (TCR) β gene undergoes rearrangements and becomes expressed. The $CD44^-CD25^-$ cells differentiate further into $CD4^+CD8^+$ double-positive (DP)

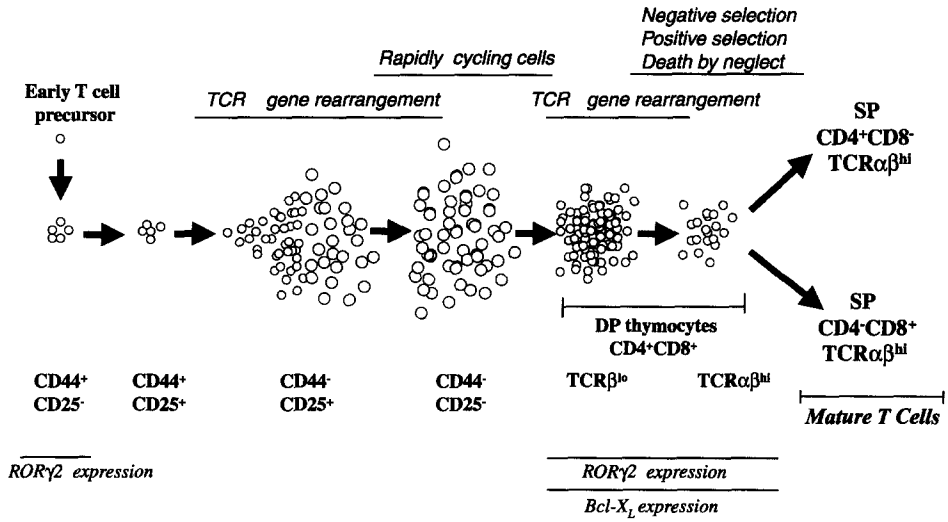

FIG. 6. Schematic of the multistep pathway of thymocyte maturation. The expression of RORγ and Bcl-X_L is indicated.

thymocytes which constitute the majority (80–85%) of the thymocyte population. At this stage TCRα rearrangements take place and TCRα begins to be expressed. A majority of DP thymocytes, which do not recognize complexes of major histocompatibility antigen complex (MHC) proteins and peptides, undergo apoptosis (death by neglect). Negative selection eliminates, via apoptosis, DP thymocytes that express self-reactive T cell antigen receptors, while thymocytes exhibiting low affinities for MHC–peptide complexes undergo positive selection. Only a small fraction of the surviving, positively selected DP cells mature further into single-positive (SP) $CD4^+$ helper and $SPCD8^+$ cytotoxic lineages. This differentiation depends critically on the specificity of the interactions between TCRs and class I and II major histocompatibility complexes. DP cells differ from SP cells in several ways. DP cells express much lower levels of TCR and do not proliferate or produce IL-2 after stimulation with anti-CD3 antibodies or calcium ionophore. In the last stage, SP thymocytes locate to the thymic medula and are released into the blood.

RORγ has been reported to be expressed at specific stages during thymopoiesis (Fig. 6). RORγ2 is expressed at high levels only in the very immature $CD44^+CD25^-$ thymocyte precursor cells and immature DP thymocytes and is undetectable in fully mature $SPCD4^+$ and $SPCD8^+$ cells (*39, 42*). These results indicate that RORγ is induced when $CD44^-CD25^-$ pre-T cells differentiate into DP thymocytes. Evidence has been provided that suggests that the induction of RORγ expression may be mediated by the pre-TCR signaling cascade (*40*). This induction precedes the expression of TEA (T early alpha). Interestingly, the TEA promoter contains an RORE able to bind RORγ2, suggesting that RORγ may regulate TEA and, as a consequence, Vα-to-Jα rearrangements (*40*). $CD4^+CD8^{low}$HSA(heat-stable antigen)^{high} cells, which are an intermediate step in the maturation from DP to $SPCD4^+$ HSA^{low} thymocytes, express moderate levels of RORγ2. The latter suggests that RORγ2 is downregulated gradually during thymocyte maturation. These observations show that RORγ2 expression is tightly regulated during thymopoiesis and suggest that RORγ controls gene expression at very specific stages of thymopoiesis.

Study of the various CD4/CD8 subpopulations in thymi from ROR$\gamma^{-/-}$ mice showed that the percentages of DP, $SPCD4^+$, and $SPCD8^+$ cells are significantly reduced compared to those of wild-type mice (*43, 44*). Although the percentage of DN cells is greatly enhanced, their total number is not changed significantly. In addition, little change is observed in the expression of CD44 and CD25, indicating that the early stages of thymopoiesis proceed normally.

The reduction in DP, $SPCD4^+$, and $SPCD8^+$ thymocytes in ROR$\gamma^{-/-}$ mice could be due to changes in differentiation, proliferation, and/or apoptosis. $SPCD4^+$ cells, although dramatically reduced in number, contain normal levels of TCR and CD4, suggesting that they have undergone positive selection. Examination of tissue sections stained by eosin/hematoxylin revealed the presence of

an increased number of apoptotic cells in thymi of ROR$\gamma^{-/-}$ mice (*43, 44*). This was confirmed by the observed increase in TUNEL staining, a measurement of the extent of DNA fragmentation. TUNEL staining is localized to the cortical regions where the DP thymocytes reside. Flow cytometric analysis confirmed that accelerated apoptosis is associated with the DP thymocytes (*44*). Crosses between ROR$\gamma^{-/-}$ mice and TCR$\alpha^{-/-}$ mice, in which DP cells are unable to undergo negative selection, demonstrated that TCR$\alpha^{-/-}$/ROR$\gamma^{-/-}$ thymocytes have the same phenotype as ROR$\gamma^{-/-}$ thymocytes, suggesting that accelerated apoptosis in ROR$\gamma^{-/-}$ thymocytes is not due to enhanced negative selection but to increased death by neglect (*43*).

Apoptosis is a multistep process of programmed cell death in which dissipation of mitochondrial transmembrane potential, release of cytochrome c, and activation of caspases, a family of cysteine proteases, often are important events (*134–137*). Caspases can be involved in the initiation of apoptosis as well as in its execution (*138, 139*). Activation of caspases leads to cleavage of various protein substrates, DNA fragmentation, and translocation of phosphatidylserine from the inner layer of the plasma membrane to the outer layer. The latter can be monitored by measuring the binding of FITC-conjugated annexin V, a Ca^{2+}-binding protein with high affinity for phosphatidylserine, by flow cytometry (*140*). Analysis of caspase activity and annexin V binding have confirmed that ROR$\gamma^{-/-}$ thymocytes undergo apoptosis at an accelerated rate (*44, 141*). Annexin V binding studies showed that within 5 h more than 80% of cultured ROR$\gamma^{-/-}$ thymocytes are undergoing apoptosis compared to 10% of ROR$\gamma^{+/+}$ thymocytes (*43, 44*). Z-VAD-FMK, a cell-permeable peptide that at high concentrations irreversibly inhibits the activity of many caspases, effectively suppresses the progression of apoptosis in cultured ROR$\gamma^{-/-}$ thymocytes (*141*) (Fig. 7).

The release of cytochrome c from mitochondria into the cytosol is a critical step in the induction of many, but not all, apoptotic signaling pathways (*136, 137*). When released into the cytosol, cytochrome c forms a complex with apoptosis-activating factor (Apaf)-1, caspase 9, and dATP that results in the activation of caspase 9 and subsequently other downstream effector caspases, ultimately leading to cell death (*134–137*) (Fig. 7). In certain cell systems, release of cytochrome c from mitochondria appears to be controlled by members of the Bcl-2 family that includes the antiapoptotic proteins, Bcl-2, Mcl-1, and Bcl-X_L, and the proapoptotic proteins, Bax, Bak, Bad, Bid, and Bcl-X_S (*142*). Bcl-2 and Bcl-X_L inhibit release of cytochrome c from mitochondria, while proteins that promote apoptosis induce this release. Bcl-2 family members may regulate exit of cytochrome c by modulating the activity of existing channels or by forming new channels in the mitochondrial membrane (*137*). A recent study demonstrated that Bax and Bak facilitate the opening of the permeability transition pore (PTP), resulting in the collapse of the mitochondrial transmembrane

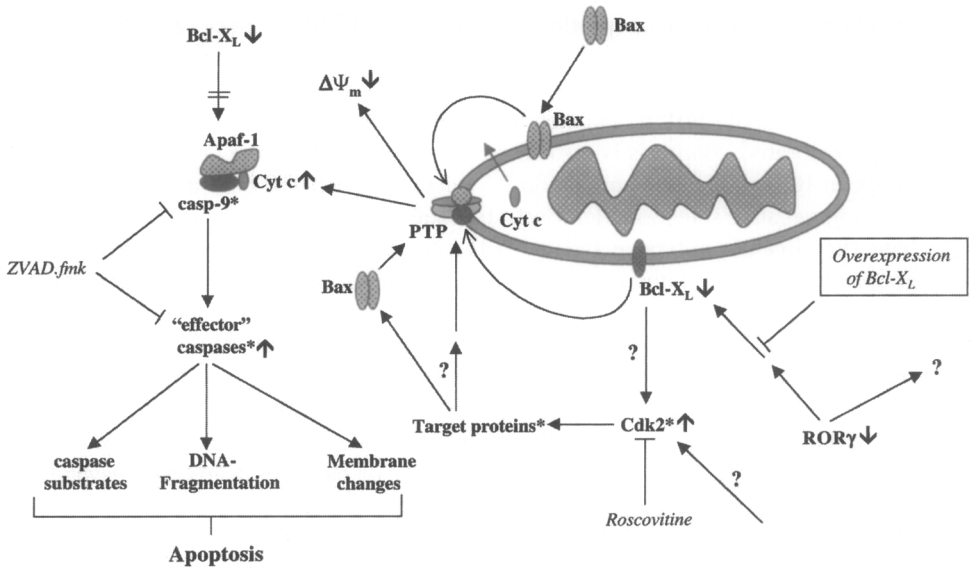

FIG. 7. Model of apoptotic events in ROR$\gamma^{-/-}$ thymocytes. ROR$\gamma^{-/-}$ thymocytes undergo accelerated apoptosis that is associated with repression of Bcl-X_L, activation of cdk2, translocation of Bax from the cytosol to the mitochondria, dissipation of $\Delta\Psi_m$, release of cytochrome c into the cytosol, activation of caspase activity, increased annexin V binding, and ultimately DNA fragmentation. The precise relationship between several of these events has not not yet been determined, and what is presented is a possible chain of events. Inhibition of caspase activity by ZVAD.fmk inhibits the execution of apoptosis. Activation of cdk2 appears to play a critical role in this induction of apoptosis, since the cdk2 inhibitor roscovitine inhibits apoptosis. Cdk2 activation is upstream from the dissipation of $\Delta\Psi_m$, release of cytochrome c, and caspase activation. Cdk2 may induce phosphorylation and thereby change the activity of proteins involved in the apoptotic process, such as Bax or p53. The link between Bcl-X_L repression and cdk2 activation is not known. Activation of cdk2 could be downstream of Bcl-X_L repression. This appears to be supported by observations showing that overexpression of Bcl-X_L blocks apoptosis and confers on ROR$\gamma^{-/-}$ thymocytes a normal cell cycle behavior. Alternatively, cdk2 activation might occur independently of Bcl-X_L and act synergistally with the reduction in Bcl-X_L. Repression of Bcl-X_L may result in change of activity of several proteins, including PTP and Apaf-1.

potential ($\Delta\Psi_m$) and possibly permeation of cytochrome c through the channel, while Bcl-X_L stimulates closure of this channel (*143*). The modulation of this channel may be mediated through interactions of Bcl-X_L, Bax, and Bak with the voltage-dependent anion channel (VDAC), one of the components of the PTP. However, release of cytochrome c can also occur in the absence of a collapse in $\Delta\Psi_m$ (*144*), suggesting the existence of other targets for members of the Bcl-2 family. The latter is illustrated by a report showing that Bcl-X_L can physically interact with Apaf-1 and inhibit maturation of caspase 9 (*145*). Clearly, the

relationships between the various proteins and activities that have been associated with apoptosis are still very controversial and await further study.

Although the precise role of RORγ in the control of apoptosis is not yet fully understood, RNA protection assays as well as Northern and Western blot analyses have shown that the expression of Bcl-X_L mRNA and protein is dramatically reduced in ROR$\gamma^{-/-}$ thymocytes (*43, 44*). Little change in the expression of Bax, Bak, and Bcl-2 mRNA is detected. Overexpression of Bcl-X_L under the control of the *lck* proximal promoter is able to rescue ROR$\gamma^{-/-}$ thymocytes from undergoing cell death (*43*). It is interesting to note that thymocytes from Bcl-$X_L^{-/-}$ mice also exhibit decreased survival as ROR$\gamma^{-/-}$ thymocytes (*146*). These observations support a model (Fig. 7) in which downregulation of the antiapoptotic protein Bcl-X_L may be at least part of the mechanism by which accelerated apoptosis occurs in ROR$\gamma^{-/-}$ thymocytes. The loss of Bcl-X_L and the observed translocation of Bax to mitochondria in ROR$\gamma^{-/-}$ thymocytes (*141*) may facilitate opening of the PTP and subsequently result in a collapse of $\Delta\Psi_m$, release of cytochrome c, and apoptosis (Fig. 7). The rapid collapse in $\Delta\Psi_m$ and release of cytochrome c into the cytosol observed in ROR$\gamma^{-/-}$ thymocytes placed in culture are in agreement with this concept (*141*). However, the loss of Bcl-X_L may have an effect on the activity of other proteins, such as Apaf-1, as well.

Apoptosis and mitosis have many features in common (*147*). Many gene products that control the cell cycle, including p53, c-myc, and retinoblastoma (Rb) protein (*148–150*), also have an effect on the susceptibility of cells to undergo apoptosis, while gene products involved in apoptosis, such as Bcl-2 and Bax, can regulate cell growth (*151–153*). In addition to the observed increase in apoptosis in ROR$\gamma^{-/-}$ thymocytes, the percentage of thymocytes in S phase is dramatically increased from 4.4% in wild-type to 25.7% in ROR$\gamma^{-/-}$ mice (*43, 44*). Moreover, thymocytes from ROR$\gamma^{-/-}$ mice contain reduced levels of the cyclin-dependent kinase (cdk) 2 inhibitor $p27^{kip1}$ while cdk2 activity is dramatically increased (*43*). These changes are probably only in part due to the increase in the percentage of rapidly proliferating $CD44^-CD25^-$ thymocytes observed in ROR$\gamma^{-/-}$ mice, since inhibition of cdk2 activity by roscovitine greatly reduces apoptosis in ROR$\gamma^{-/-}$ DP thymocytes. These observations indicate not only that the increase in cdk2 activity is associated with apoptosis but that cdk function is required for the accelerated apoptosis in ROR$\gamma^{-/-}$ DP thymocytes (*43*) (Fig. 7). Activation of cdk2 has been reported to play a critical role in the induction of apoptosis in thymocytes by a number of apoptotic stimuli (*153, 154*). These studies indicated that activation of cdk2 acts upstream of caspases and the $\Delta\Psi_m$, and demonstrated that it is a step of no return. However, these studies reached different conclusions about whether cdk2 acts up- or downstream of p53 and Bcl-2. The activation of cdk2 in ROR$\gamma^{-/-}$ DP thymocytes also

occurs upstream of caspases and $\Delta\Psi_m$ (Fig. 7); however, what its relationship is with the downregulation of Bcl-X_L has not yet been established. Expression of Bcl-X_L in ROR$\gamma^{-/-}$ thymocytes restores normal cell cycle behavior and inhibits apoptosis (*43*), suggesting that Bcl-X_L may function upstream of cdk2 activation. Alternatively, activation of cdk2 may be induced independently of Bcl-X_L when thymocytes are placed in culture and act synergistically with the downregulation of Bcl-X_L. The inhibition of apoptosis in ROR$\gamma^{-/-}$ thymocytes by roscovitine may support the latter hypothesis. One mechanism by which cdk2 might induce apoptosis is through phosphorylation of other apoptosis-regulatory proteins, such as proteins that control PTP function or caspase activation (*153*) (Fig. 7). Bax and p53 have been proposed as possible target proteins for cdk2 phosphorylation.

Repression of Bcl-X_L expression observed in ROR$\gamma^{-/-}$ thymocytes might imply that the inverse could also be true. Expression of RORγ may induce Bcl-X_L expression and thereby function as a suppressor of apoptosis and enhance survival of thymocytes. The expression of Bcl-X_L during thymopoiesis has been reported to be restricted to DP thymocytes (*146, 155*) (Fig. 6) and therefore is coexpressed with RORγ2 in the same cells. Whether Bcl-X_L is a direct target gene for RORγ or whether RORγ regulates Bcl-X_L expression by an indirect mechanism has yet to be established (Fig. 7). Examination of the 750-bp 5′-regulatory region of the Bcl-X_L gene has not identified any sequence resembling an RORE.

RNase protection analysis showed relatively little change in the expression of Fas or FasL, suggesting that the increased apoptosis in ROR$\gamma^{-/-}$ thymocytes is not due to increased FasL expression (*44*). This is supported by observations showing that gld/gld/ROR$\gamma^{-/-}$ thymocytes obtained from ROR$\gamma^{-/-}$ mice crossed with glg/gld mice defective in FasL function, underwent apoptosis to the same extent as ROR$\gamma^{-/-}$ thymocytes (*43*). In addition, in contrast to apoptosis in ROR$\gamma^{-/-}$ thymocytes, induction of apoptosis by FasL cannot be blocked by Bcl-2 or Bcl-X_L (*156*).

VIII. Overexpression of RORs

A. Effect of RORγ on Thymopoiesis and Apoptosis

He and coinvestigators used a different approach to study the role of RORγ2 in thymopoiesis (*42*). Transgenic mice were generated in which the ectopic expression of RORγ2 was driven by the hCD2 promoter/enhancer regulatory region. This promoter targets expression of RORγ2 to all T cells, immature as well as mature. Therefore, in these RORγ2 transgenic mice, RORγ2 can be detected in several DN subpopulations, SPCD4$^+$ and SPCD8$^+$ cells, and in

T lymphocytes of spleen and lymph nodes where RORγ2 normally is not expressed. The number of thymocytes in RORγ2 transgenic mice is reduced by 85%. The percentage of DP thymocytes is dramatically lower than in their wild-type littermates while the percentage of DN thymocytes increases. Although the percentage of $SPCD4^+$ and $SPCD8^+$ thymocytes is higher in transgenics, the absolute number of these cells is significantly reduced compared to control mice. The number of T lymphocytes in spleen and lymph nodes is also reduced, by 50–60%. These observations indicate that targeted expression of RORγ2 affects the transition of DN to DP thymocytes and suggest that downregulation of RORγ during early thymopoiesis is essential for normal thymocyte differentiation to proceed.

Analysis of triple-negative (TN) $CD4^-CD8^-CD3^-$ cells showed several changes in the distribution of the different TN subpopulations from RORγ2 mice compared to those from control mice. The percentage of $CD44^-CD25^+$ cells is dramatically enhanced, whereas few $CD44^-CD25^-$ cells are detected. The changes in these two cell populations could be attributed to an inhibition of the differentiation of the $CD44^-CD25^+$ subpopulation into $CD44^-CD25^-$ cells, to an inhibition of the proliferation and expansion of the $CD25^-CD44^-$ subpopulation, or to increased apoptosis in $CD25^-CD44^-$ cells (*42*). The last possibility can be ruled out, because no increase in apoptosis is observed. Cell cycle analysis showed that less than 3% of the $CD44^-CD25^-$ subpopulation from RORγ2 mice is in the S and G2/M phase of the cell cycle compared to 30% of the $CD44^-CD25^-$ cells from control mice. These observations strongly indicate that ectopic expression of RORγ2 in these transgenic mice inhibits the proliferation of $CD44^-CD25^-$ thymocytes. In addition to inhibiting $CD44^-CD25^-$ cells, ectopic expression of RORγ2 also suppresses proliferation of mature $SPCD4^+$ cells by phorbol ester and ionomycin.

Ectopic expression of RORγ2 has an effect on the expression of several genes. The expression of TCR in SP thymocytes and peripheral T cells from spleen and lymph nodes is downregulated in RORγ2 transgenic mice while FasL expression is only slightly affected (*42*). The induction of IL-2 by PMA and ionomycin is 3–6-fold lower in $SPCD4^+$ and splenic T cells from RORγ2 transgenic mice compared to those of wild-type mice. The regulation of IL-2 is complex and has been reported to involve multiple transcription factors, including Erg family members and c-rel (*157*). Although ectopic expression of RORγ2 negatively regulates c-rel expression, ectopic expression of c-rel is unable to reverse the effect of RORγ2, indicating that downregulation of interleukin 2 by RORγ2 does not involve c-rel or involves other factors in addition to c-rel. Similar results were obtained in T cell hybridoma KMIs-8.3.5 cells overexpressing RORγ2 (*39*).

As mentioned above, study of $ROR\gamma^{-/-}$ mice has indicated that RORγ expression suppresses apoptosis in thymocytes. Such a negative regulatory role has

also been observed in T cell hybridomas. Ectopic expression of RORγ in T cell hybridoma cells DO11.10, 2B4, and KMIs-8.3, which normally do not express RORγ, greatly inhibits the induction of apoptosis (*39*) (S. Kurebayashi and A. M. Jetten, unpublished observations). T cell hybridomas expressing RORγ become refractory to both TCR-mediated apoptosis by anti-CD3 monoclonal antibodies and TCR-independent apoptosis stimulated by phorbol ester plus ionomycin. However, the induction of apoptosis by FasL, dexamethasone, ceramide, and the kinase inhibitor staurosporin is not affected by RORγ (*39*). These results indicate that RORγ affects specific apoptotic signaling pathways in T cells. To induce this inhibitory effect, both the DBD and the LBD of RORγ are required. RORγ2 was shown to be more effective in inhibiting apoptosis than was RORγ1.

TCR-mediated apoptosis is a complex, multistep process that, through the activation of various kinases and phosphatases, results in the activation and increased expression of several transcriptional factors (*158*). These factors include the nuclear orphan receptor Nur77 (*159–161*), members of the nuclear factor of activated T cells (NFAT) (*162, 163*), the forkhead family (*164*), and the Egr family (*165, 166*). The induction of these transcription factors leads to an increase in the transcription of several other genes, including Fas-ligand and interleukin 2. FasL is secreted and, after binding to the Fas receptor, induces T cell death. Since the induction of apoptosis by FasL is not inhibited by RORγ, the inhibition of apoptosis by RORγ appears to occur at the level of FasL expression or at a step further upstream of this induction. Northern analysis demonstrated that the inhibition of TCR-mediated apoptosis in T cell hybridomas by RORγ is related to the repression of FasL mRNA induction. The antagonism of TCR-mediated apoptosis in T cell hybridomas by retinoic acid or glucocorticoids must not be mediated by RORγ, because studies have shown that RORγ is not induced by these agents (*39*). RORγ also inhibits the TCR-mediated increase in IL-2 mRNA but does not affect the induction of CD69 and CD44, indicating that RORγ inhibits a specific step that may be common to the control of FasL and IL-2 expression. Interestingly, some of the same transcription factors, including members of the Egr family, have been implicated in the regulation of both of these genes.

The mechanism by which RORγ suppresses FasL induction remains to be elucidated. The regulation of FasL gene expression is complex and many transcription factors have been implicated in its control. Activation of TCR results in a dramatic increase in the expression of Nur77, Egr2, and Egr-3 mRNAs (*39*). However, RORγ does not inhibit the induction of these transcription factors, suggesting that the repression of FasL by RORγ occurs at a different level downstream of this induction. Preliminary results have indicated that RORγ can suppress Egr-mediated transactivation, suggesting that antagonism between the RORγ and Egr signaling pathways may be responsible for repression of FasL expression by RORγ (M. Sakaue and A. M. Jetten, unpublished observations).

B. Inhibition of Myogenesis by Dominant-Negative RORα

RORα has been reported to be expressed in skeletal muscle tissue and in the mouse myoblast cell line C2C12 (*25, 167*). Its expression does not change when proliferative C2C12 cells undergo differentiation into postmitotic, multinucleated myotubules upon serum withdrawal. To examine the role of RORα in muscle differentiation, a dominant-negative RORα (dn-RORα) expression vector was stably transfected into C2C12 cells and its effect on myogenesis was determined (*167*). These results showed that ectopic expression of dn-RORα delays and inhibits muscle cell differentiation. Forty-eight hours after serum withdrawal, C2C12 cells expressing dn-RORα do not express skeletal myosin heavy chain (MHC) or form myotubules, in contrast to parental cells. However, MHC and myotubules appear after 96 h, although not to the same level as in control cells. Expression of dn-RORα inhibits the induction of both MyoD and myogenin, two basic helix–loop–helix transcription factors critical in the control of myogenesis. The dn-RORα also inhibits the induction of the cdk-inhibitor $p21^{WAF1/Cip1}$, a marker for cell cycle exit. Based on this inhibitory function of dn-RORα, it was concluded that RORα may positively regulate myogenesis. This study also provided evidence for a direct interaction between RORα and MyoD (*167*). This interaction requires the amino-terminal activation domain of MyoD and the DBD of RORα. The precise role this interaction has in the control of myogenesis is yet to be determined.

IX. Other Target Genes

A number of potential ROR target genes have been identified. These include 5-lipoxygenase, γF-crystallin, apoA-I, laminin B1, cellular retinol binding protein (CRBP), oxytocin, Purkinje cell protein-2, TEA, and $p21^{WAF1}$ (*32, 40, 76, 113, 168–170*). The identification of these target genes is based largely upon the presence of ROREs in their 5′-promoter flanking regions. However, little evidence has been accumulated to indicate that these genes are true targets for RORs in a physiological setting. The regulation of TEA has already been discussed above.

The promoter of the gene encoding the neuropeptide oxytocin has been found to be activated by RORα (*170*). This gene is expressed in specific hypothalamic neuroendocrine cells, the pineal gland, the uterine epithelium, fetal membranes, and corpus luteum, all sites of high RORβ expression. Two RORE-like elements have been found in the oxytocin promoter region. Mutations in these elements significantly reduced transcriptional activation by ROR. A single RORE has been identified in both the 5-lipoxygenase and mCRBP promoter.

Gel shift analyses demonstrated binding of RORα to these DNA elements (*168*). In *Drosophila* SL-3 cells, RORα was able to enhance transcription of a reporter under the control of these elements. The RORE in the 5-lipoxygenase was able to bind RORα1 but not RORα2 or RORα3. Although studies with promoter–reporter constructs and cotransfection with ROR expression vectors can demonstrate that ROR is able to bind to these sites, they do not prove that these genes are truly targets for ROR *in vivo*.

The laminin B1 gene contains three core motifs spaced by 3 and 13 bp. All three core motifs are necessary to confer ROR- and RAR-dependent transactivation (*169*). Cotransfection of a reporter under the control of the −460-bp promoter flanking region of the laminin B1 gene along with an RORα1 expression vector resulted in a sevenfold increase in reporter activity. The activation was inhibited by cotransfection with RAR. This inhibition is likely due to competition for binding to the same elements.

RORα1 has been reported to enhance the transcriptional activation mediated by the apoA-I promoter in the colon carcinoma Caco-2 cells (*113*). In this study, neither RORα2 nor RORα3 was able to induce transcriptional activation through the apoA-I promoter. An ATATATAGGTCA sequence was found that overlapped with the TATA-box. Mutation of the AGGTCA core motif abolished the transactivation by RORα1. EMSA showed that RORα1 could bind to the wt-RE but not to the mutated RE. To analyze the physiological significance of this regulation the level of apoA-I expression in *wt* and *sg/sg* mice were compared. Results showed that the level of apoA-I mRNA was significantly lower in the intestine of *sg/sg* mice than that of *wt* mice. These observations appear to support the hypothesis that the APO-AI gene is a target gene for RORα1 and indicate a potential role for RORα in the regulation of genes involved in lipid and lipoprotein metabolism. The severe atherosclerosis observed in *sg/sg* mice kept on a high-fat diet is in agreement with such a concept.

Scanning the databases for the presence of RORE sites in sequences involved in transcriptional regulation revealed an RORE in the first intron of the N-Myc gene, a region reported to be implicated in the control of this gene (*65*). In further studies, EMSA demonstrated that RORα was able to bind to this RORE site. In addition, RORα was shown to induce transcriptional activation of N-Myc significantly when an RORα expression vector and the entire N-Myc transcription unit of 7.3 kb were cotransfected into COS-7 cells. The inhibition of N-Myc induction during differentiation of embryonal carcinoma P19 cells by the nuclear receptor Rev-Erbβ was reversed by RORα. This antagonism is likely due to competition between the two receptors for the same response element. N-Myc in combination with activated Ha-ras causes oncogenic transformation in rat embryo fibroblasts and an increase in the formation of transformed foci. Concomitant expression of RORα causes a twofold increase in foci formation,

indicating that RORα enhanced the transformed phenotype in these cells (65). These results suggest that expression of RORα may contribute to the progression of certain neoplasias.

RORα is also expressed in the murine lens. Tini *et al.* (76) have shown that RORα can activate transcription through the γF-crystallin promoter. An RE was identified between nucleotides −210 to −185 of the γF-crystallin promoter that can bind either a ROR monomer or a RAR/RXR heterodimer, which suggests that these receptors compete with each other for binding to this element. The constitutive activation of this element by ROR can be suppressed by the RAR/RXR heterodimer. However, further studies must discern whether this antagonism has any physiological significance and whether γF-crystallin is indeed a true target gene for ROR.

X. Concluding Remarks

It is clear from the studies reviewed above that much progress has been made in understanding the mechanisms of action and biological functions of RORs. However, these studies have also left open some questions and raised new ones. The unique pattern of expression of the various ROR isoforms suggests that each isoform is under a different transcriptional control and regulates different physiological processes. These studies have also demonstrated that RORs play critical roles in the regulation of a number of physiological processes, including motor coordination, circadian rhythm, bone metabolism, thymopoiesis, apoptosis, and lymph node development. Future studies must determine the exact role of RORs in these biological processes and the precise mechanisms and target genes by which RORs regulate these processes. For example, they have to determine the molecular mechanism by which cdk2 activity is induced and Bcl-X_L is repressed in ROR$\gamma^{-/-}$ thymocytes, what the relationship is between these two changes, and how they relate to the induction of apoptosis. Further characterization of the molecular mechanisms of action of RORs will not only provide greater insight into their functions in normal physiological processes but will also determine whether RORs are implicated in any diseases. Recent studies have provided preliminary evidence for a potential role of RORs in atherosclerosis and immune disorders. It has also become evident that RORs do not act as constitutively active receptors but that their activities are regulated by a mechanism that could involve ligands and/or specific kinases. The link between CaMKIV and ROR activation is intriguing and suggests that the activity of RORs may be under the control of signaling pathways that regulate Ca^{2+} concentration. Future study of the mechanisms that control ROR activation may lead to the development of novel therapeutic strategies.

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