Identification of Natural Monomeric Response Elements of the Nuclear Receptor RZR/ROR

They also bind COUP-TF homodimers

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The receptor RZR/ROR is an important member of the nuclear receptor superfamily and has recently been shown to be the nuclear receptor for the pineal gland hormone melatonin. RZR/ROR binds as a monomer to DNA, and the human 5-lipoxygenase gene has been identified as the first RZR/ROR/melatonin-responding gene. Another prominent nuclear receptor is COUP-TF, which binds as a dimer to DNA. In this study, the sequences of known promoter regions of genes that may be involved in the physiological action of melatonin have been screened for putative monomeric RZR/ROR response elements. The binding of RZR/ROR and COUP-TF was compared and quantified on a set of 12 putative response elements. Interestingly, COUP-TF homodimers were found to bind with high affinity to some of the monomeric RZR/ROR response elements. Four RZR/ROR response elements, found in the genes of the mouse bifunctional enzyme, the rat bone sialoprotein, mouse Purkinje cell protein 2, and human p21WAF1/CIP1, were shown to be inducible by melatonin under conditions of low constitutive activity. Surprisingly, the constitutive activity of COUP-TF was also stimulated by an unknown serum compound. The novel Purkinje cell protein 2 and p21WAF1/CIP1 RZR/ROR/melatonin-responding genes may be the key for understanding the role of RZR/RORα in the mouse mutation staggerer and the antiproliferative action of melatonin, respectively.

Many important physiological functions are controlled at the level of transcriptional regulation by members of the nuclear receptor superfamily. This family of structurally related transcription factors contains the nuclear receptors for steroid hormones (aldosterone, cortisol, estrogen, progesterone, and testosterone), 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), thyroid hormone, and all-trans-retinoic acid (RA), but also >100 orphan nuclear receptors, for which no ligand was known at the time of their discovery (1).

Nuclear receptors activate transcription through DNA sequences in the promoter region of target genes, referred to as response elements. Since the DNA-binding domain is highly conserved in all nuclear receptors (2), they bind in a vast majority to the hexameric consensus motif AGGTCA (3). However, most of them do not have sufficient affinity to bind to DNA as a monomer, so they have to form homodimers or heterodimers with another member of the superfamily (4). Therefore, most natural response elements are formed by two hexameric motifs in a directly repeated or inverted palindromic orientation. Specificity in the recognition of these response elements is mainly obtained by the number of spacing nucleotides (5, 6). In contrast, nuclear receptors that are able to bind as a monomer increase the specificity of their interaction with DNA through the recognition of two to four nucleotides 5’-flanking the hexameric consensus motif (7).

RZR/ROR belongs to those nuclear receptors that bind DNA as a monomer (8, 9). To date, three RZR/ROR subtypes are known: RZR/RORα is rather ubiquitously expressed (10), whereas RZR/RORβ is brain-specific (8, 11), and the highest expression of RZR/RORγ is found in skeletal muscle (12). RZR/ROR gained special attention through the finding that the pineal gland hormone melatonin is a specific ligand (11, 13). So far, only for two other orphan nuclear receptors have natural ligands been identified: 9cis-RA for the retinoid X receptor (14, 15) and 15-deoxy-Δ12,14-prostaglandin J₂ for peroxisome proliferator-activated receptor-γ (16, 17).

Melatonin has pleiotropic physiological functions, which are in part not well understood, especially with respect to the underlying molecular mechanisms (18). Experimental evidence for nuclear melatonin signaling has already been reported some years ago (19–22), and the discovery of RZR/ROR as the nuclear melatonin receptor provides a mechanism as to how the hormone mediates transcriptional regulation. The identification of novel RZR/ROR- and melatonin-responding genes will allow an improved understanding of melatonin’s action. The first example was the discovery of the repression of 5-lipoxygenase mRNA expression in human B lymphocytes by melatonin via RZR/RORα (23), which predicted a potential anti-inflammatory role for the pineal gland hormone (24).

Another interesting nuclear receptor is the ubiquitously expressed COUP-TF (chicken ovalbumin upstream promoter transcription factor) (25), which is known to repress several nuclear hormone signaling pathways by competition with other nuclear receptors for binding to their response elements (26, 27). COUP-TF has been shown to form homodimers and heterodimers with the retinoid X receptor on dimeric response elements. In contrast to the other members of the nuclear receptor superfamily, COUP-TF does not show a clear response element preference (25, 28).

The studies described here were undertaken to characterize novel RZR/ROR response elements that have been identified by screening sequences of known hormone response elements and...
promoter regions. The binding of RZR/ROR and COUP-TF was compared and quantified on a set of 12 putative response elements. The four most interesting response element candidates, which were found in the mouse BFE, rat BSP, mouse PCP-2, and human p21<sup>WAF1/CIP1</sup> genes, were functionally characterized.

**MATERIALS AND METHODS**

DNA Constructs—For each of the 12 monomeric response elements (core sequence given in Fig. 1), an oligonucleotide with the constant 5'-and 3'-attaching sequences ATTTCATG and TCTAGAC, respectively, was synthesized and annealed with the oligonucleotide GGGTCTA-GATGACC. The semi-double-stranded DNA fragments were filled in with Klenow polymerase (Promega), digested with XbaI (Promega), and subcloned into the XbaI site of pBlCAT2 (29). In this way, they were fused to the thymidine kinase (tk) promoter to drive the expression of the chloramphenicol acetyltransferase (CAT) reporter gene. Rat RZR/ ROR<sub>β</sub>, chicken T<sub>R</sub>R<sub>α</sub>, human COUP-TF, and human RZR/ROR<sub>α</sub> DNAs were subcloned into the expression vector pSG5 (Stratagene).

*In vitro* Translation and DNA Binding Assays—Linearized DNAs encoding RZR/ROR<sub>β</sub>, T<sub>R</sub>R<sub>α</sub>, COUP-TF, and RZR/ROR<sub>α</sub> were used for in vitro transcription as recommended by the supplier (Promega). For *in vitro* translations, 10 μl of each RNA were mixed with 175 μl of rabbit reticulocyte lysate, 100 units of RNasin, and 20 μM complete amino acid mixture (all from Promega) in a total volume of 250 μl and incubated at 30 °C for 180 min. The response element probes were prepared by using 200 ng of the respective semi-double-stranded DNA fragments, 10 μCi of [α-<sup>32</sup>P]dCTP (3000 Ci/mmol; DuPont NEN), and Klenow polymerase. Labeled response elements were purified using BioSpin6 columns (Bio-Rad) and quantified for specificity of radiolabeling by scintillation counting. Per DNA binding assay, 5 μl of in vitro translated receptors were incubated with 1.3 ng of response element probe in a total volume of 20 μl of binding buffer (10 mM Hepes (pH 7.9), 40 mM KCl, 1 mM dithiothreitol, 0.2 μg/μl poly(dI-dC), and 5% glycerol) for 20 min at room temperature. For Scatchard analysis, 5 μl of in vitro translated RZR/ROR<sub>α</sub> or COUP-TF, were incubated with eight different amounts of the respective response element probe ranging from 0.016 to 0.67 ng as described (30). Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels (at room temperature) in 0.5 × Tris/boric acid/EDTA (45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8.3)). After exposure to a film to localize the free probe and protein-probe complexes, the respective bands were excised and scintillation-counted.

Cell Culture, Transfection, and CAT Assays—Drosophila SL-3 cells (2 × 10<sup>4</sup> cells/well in a 6-well plate) (31) were grown overnight in Schneider’s medium (Life Technologies, Inc.) without fetal calf serum (FCS). Liposomes were formed by incubating 2 μg of CAT reporter constructs, 1 μg of receptor expression vector, and 1 μg of reference plasmid pCH110 (Pharmacia Biotech Inc.) with 11 μg of N-(I-2,3-dihydroxypropyl)-N,N-trimethylammonium methylsulfate (Boehringer Mannheim) for 15 min at room temperature in a total volume of 100 μl. After dilution with 0.9 ml of Schneider’s medium, the liposomes were added to the cells. 4-8 h after transfection, 500 μl of Schneider’s medium supplemented with or without 30% FCS (Life Technologies, Inc.), 1 μg melatonin (Fluka), or solvent (dimethyl sulfoxide) were added. After an additional 16 h, the cells were harvested, and CAT assays were performed as described (32). The CAT activities were normalized to β-galactosidase activity; each condition was analyzed at least in triplicate, and the data are shown as means ± S.D.

**RESULTS**

Three natural RZR/ROR response elements have been described to date. The RA response elements in the promoters of the mouse cellular retinol-binding protein I (CRBP-I) (33) and chicken γ<sup>F</sup>-crystallin (34) genes are overlaid with a binding site for RZR/ROR. Competition between RA receptor (RAR)-retnoid X receptor heterodimers and RZR/ROR monomers has been shown on both response elements (8, 35). However, the first monomeric RZR/ROR response element has been identified in the promoter of the human 5-lipoxygenase gene (23). All three elements are characterized by the hexameric consensus motif RGGTCA (R = A or G), but their 5' preceding sequence is divergent (Fig. 1). Analysis of synthetic RZR/ROR response elements (8, 9) indicated that a T in position −1 and an A in position −4 in front of the hexameric core motif are important for high affinity binding of the receptor; Fig. 1 shows a synthetic RZR/ROR response element (syn) that has been used in several previous studies (8, 11, 13, 35).

A screening of ~100 natural dimeric response elements for RA, 1,25-(OH)<sub>2</sub>D<sub>3</sub>, thyroid hormone, and peroxisome proliferator-activated receptors showed that only a minority contain perfect RGGTCA motifs. Moreover, even if a T in position −1 should statistically be expected in 25% of the cases, it was found in front of <10% of the RGGTCA motifs. In addition to the RA response element of the CRBP-I gene, a TRGGTCA motif was found only in the putative 1,25-(OH)<sub>2</sub>D<sub>3</sub> response element of the rat BSP gene (36) and even in two sites of the complex peroxisome proliferator-activated receptor response elements of the BFE gene (Fig. 1) (37, 38). A systematic screening of the promoter sequences of ~50 genes, whose gene products are known to have important cell regulatory functions, identified putative RZR/ROR response elements in the human BSP (39), human RAR<sub>β</sub> (40), mouse PCP-2 (41), and human and mouse p21<sup>WAF1/CIP1</sup> genes (Fig. 1).

Gel shift assays were performed with equal amounts of the synthetic and the 11 natural monomeric RZR/ROR response elements (see Fig. 1) and in vitro translated human RZR/ ROR<sub>α</sub>, rat RZR/ROR<sub>β</sub>, chicken T<sub>R</sub>R<sub>α</sub>, and human COUP-TF (Fig. 2). In confirmation of previous results (8), RZR/ROR<sub>α</sub> and RZR/ROR<sub>β</sub> displayed identical response element preference. Both subtypes were shown to bind with high affinity as a monomer to the synthetic response element and to the response elements of the mouse CRBP-I, human RAR<sub>β</sub>, and rat BSP, human p21<sup>WAF1/CIP1</sup>, and mouse PCP-2 genes; with reasonable affinity to the response elements of the mouse BFE (both sites), human 5-lipoxygenase, and mouse p21<sup>WAF1/CIP1</sup> genes; and with low affinity to the response elements of the human BSP and chicken γ<sup>F</sup>-crystallin genes. T<sub>R</sub>R<sub>α</sub> monomers showed low affinity binding to the response elements of the human RAR<sub>β</sub>, 5-lipoxygenase, and BSP genes, but no binding to the remaining nine response elements. Surprisingly, COUP-TF...
was found to bind as a homodimer (according to equal migration of other dimeric nuclear receptor complexes on dimeric response elements) to the monomeric response elements. COUP-TF homodimers also showed a response element preference, which was in part different from that of RZR/ROR monomers. COUP-TF homodimers were found to bind with high affinity to the synthetic response element and to the response elements of the mouse BFE (site 2), mouse CRBP-I, rat BSP, and mouse PCP-2 genes; with lower affinity to the response elements of the human RARβ, 5-lipoxygenase, BSP, and p21WAF1/CIP1 genes; and with very low affinity to the response elements of the mouse BFE (site 1), chicken γF-crystallin, and mouse p21WAF1/CIP1 genes.

These rough classifications of receptor-DNA affinities were based on the visual impression of several independently performed gel shift assays. However, to compare quantitatively the binding affinity of RZR/RORα monomers and COUP-TF homodimers for the same monomeric response element, the respective dissociation constants (Kd) were determined. Due to the rather low receptor affinity of the response elements of the mouse BFE (site 1), human BSP, and chicken γF-crystallin genes, only the nine remaining response elements were analyzed. Constant amounts of receptor protein were incubated with eight different concentrations of each response element probe, and the protein-bound probe was quantified in comparison to the free probe. The ratio between the bound and free probes was plotted in respect to the concentration of the bound probe and allowed us to calculate the respective Kd values (Fig. 3). The Kd value for RZR/RORα on the synthetic response element (1.2 nM) is in good agreement with the previously determined value (1.6 nM) (8). The response elements of the rat BSP (0.6 nM), mouse PCP-2 (0.6 nM), and mouse CRBP-I (0.9 nM) genes showed even higher affinity for RZR/RORα than the synthetic response element, whereas the response element of...
the human \( p21^{WAF1/CIP1} \) gene (1.2 \( \text{nM} \)) showed the same affinity for RZR/ROR\( \alpha \). The four remaining response elements showed clearly lower affinities for the receptor (\( K_d \) values between 2.0 and 2.3 \( \text{nM} \)). In contrast, the monomeric response element of the mouse BFE gene (site 2) showed the highest affinity (0.8 \( \text{nM} \)) for COUP-TF homodimers, followed by the synthetic response element (0.9 \( \text{nM} \)) and the response elements of the mouse PCP-2 (1.0 \( \text{nM} \)), rat BSP (1.1 \( \text{nM} \)), and mouse CRBP-I (1.2 \( \text{nM} \)) genes. The four remaining response elements (with \( K_d \) values between 1.6 and 3.2 \( \text{nM} \)) showed clearly lower affinities for COUP-TF.

The comparison of the DNA binding affinities of RZR/ROR\( \alpha \) and COUP-TF highlighted four response elements. The response elements of the rat BSP and mouse PCP-2 genes showed high affinity for both receptors, whereas those of the human \( p21^{WAF1/CIP1} \) and mouse BFE (site 2) genes displayed clear preference for RZR/ROR\( \alpha \) and COUP-TF, respectively. To test the functionality of these response elements, they were fused with the tk promoter driving the expression of the CAT reporter gene. Each of the four CAT reporter constructs was transfected together with the parental expression vector pSG5 (for control), the expression vector for RZR/ROR\( \alpha \) or the expression vector for COUP-TF into Drosophila SL-3 cells (Fig. 4). This cell line is devoid of mammalian receptors and is an established model system for the analysis of nuclear receptors (8, 13, 43). To reduce the constitutive activity of the receptors, the cells were grown for 24 h before transfection in the absence of FCS. After transfection, the cells were stimulated for 16 h either with solvent (dimethyl sulfoxide) or with melatonin (1 \( \mu \text{M} \)) both in the absence and presence of FCS (10%). In accordance with previously reported results on the synthetic response element (8, 13), also on the four natural response elements, the addition of FCS increased the constitutive activity of RZR/ROR\( \alpha \) -3-fold. Interestingly, also the constitutive activity of COUP-TF was found to be enhanced by -2-fold. In the presence of FCS, the stimulation with melatonin had no significant effect on the CAT reporter gene activity, whereas at low constitutive activity of RZR/ROR\( \alpha \), the stimulation with melatonin resulted in 3- to 5-fold induction of gene activity. In accordance with the affinity of RZR/ROR\( \alpha \) and COUP-TF for the response elements of the rat BSP and mouse PCP-2 genes, both receptors mediated high CAT activity from these elements. The preference of RZR/ROR\( \alpha \) monomers for the response element of the human \( p21^{WAF1/CIP1} \) gene and of COUP-TF homodimers for the response element of the mouse BFE (site 2) gene was also reflected at the functional level.

**DISCUSSION**

This report describes eight novel natural RZR/ROR response elements that have been identified in the promoter regions of the human and mouse \( p21^{WAF1/CIP1} \), mouse PCP-2, human and rat BSP, mouse BFE, and human RAR\( \beta \) genes. The RZR/ROR response elements of the rat BSP, mouse PCP-2, and human \( p21^{WAF1/CIP1} \) genes are of particular interest since they are bound with very high affinity by RZR/ROR\( \alpha \), they mediate high RZR/ROR\( \alpha \)-dependent constitutive gene activity, and they exist under conditions of low constitutive receptor activity inducible by melatonin.

The rat BSP RZR/ROR response element is part of a putative 1,25-(OH),\( \text{D}_3 \) response element (36), but on an extended version of the response element that contained an additional cryptic receptor binding motif (AGGGTT), no 1,25-(OH),\( \text{D}_3 \) receptor binding and no 1,25-(OH),\( \text{D}_3 \) inducibility could be detected (data not shown). However, the monomeric response element is also overlaid by an inverted TATA box (44) and provides a composite site for a putative interaction of RZR/ROR with the TATA box-binding protein. BSP is a non-collagenous protein of the extracellular matrix and is expressed in all mineralizing tissues (45), but, interestingly, also in breast cancer (46). So far, there is no indication for a role of melatonin in BSP gene regulation or in bone formation in general, but it is remarkable that both the human and rat BSP genes contain a RZR/ROR response element.

The mouse PCP-2 RZR/ROR response element is not part of any known dimeric response element, but like the rat BSP element, it is also recognized with high affinity by COUP-TF homodimers. The expression of PCP-2 is restricted to cerebellar Purkinje cells and retinal bipolar neurons (41), which both also express members of the RZR/ROR family (11). The mutation staggerer in mice is characterized by severe cerebellar ataxia, which in fact is due to a defect in the development of Purkinje cells. Very recently, this mutation was genetically mapped to a region that contains the gene for RZR/ROR\( \alpha \) (47), and it was found that staggerer mice have a deletion in this gene that...
prevents the translation of the ligand-binding domain of RZR/ RORα. PCP-2 appears to play an important role during the development of Purkinje cells (48), and disruption of PCP-2 mRNA expression caused by truncated RZR/RORα could be one explanation for the phenotype of the staggerer mutation. This link makes it very likely that PCP-2 is a physiological RZR/ROR-responding gene, and it will be very interesting to analyze the role of melatonin in this context.

The human p21
waf1/cip1
RZR/ROR response element binds COUP-TF homodimers with relatively low affinity and appears to lie in the five or six nucleotides that flank the hexameric motif RGGTCA, but differ in their 9-10 flanking sequences may form a cryptic negative regulator of the cell cycle (49, 50). The main regulator of the p21
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ROR-responding gene, and it will be very interesting to analyze the role of melatonin in this context.

The question of whether the antiproliferative effect of melatonin in the central nervous system is related to RZR/ROR, as a general repressor of the action of monomeric as well as dimeric receptors like RZR/ROR and suggests that COUP-TF may act as a general repressor of the action of monomeric as well as dimeric nuclear receptors.

A further interesting observation was that FCS enhanced the constitutive activity of COUP-TF. The serum effect for COUP-TF is not as prominent as for RZR/RORα, but it indicates that FCS may contain either a ligand or an activator for COUP-TF. To date, no ligand for COUP-TF has been identified, and it will be very interesting to analyze the role of melatonin in this context.

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COUP-TF.Todate,noligandforCOUP-TFhasbeenidentified, indicates that FCS may contain either a ligand or an activator for COUP-TF. Investigations will give further insight into the mechanisms involved in the activation of COUP-TF.