Differential Regulation of Direct Repeat 3 Vitamin D₃ and Direct Repeat 4 Thyroid Hormone Signaling Pathways by the Human TR4 Orphan Receptor*

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Steroid hormones are key physiological mediators of development and homeostasis (1–4). Understanding the crosstalk between steroid hormone-dependent and -independent signaling pathways is critical for gaining further insight into the integration of cellular regulatory cues that modulate development and tissue-specific gene expression. The biological effects of steroids and related hormones, including derivatives of vitamin D₃, are mediated through their cognate receptors (1–4). These receptors are members of a large group of ligand-activated proteins that act as transcriptional activators or repressors. However, there is another group of nuclear receptors that act as transcriptional activators or repressors. This may occur by several different mechanisms: competition for the same response element, heterodimer formation with the regulated receptor, or heterodimer formation with the retinoid X receptor (RXR); thus titrating out available RXR protein. For instance, chicken ovalbumin upstream promoter transcription factors have been demonstrated to suppress ligand-induced gene activation, including that of vitamin D₃, vitamin A, and thyroid hormone target genes (5). This interfering effect might involve the formation of a transcriptional silencing complex with RXR or competition with steroid hormone receptors for DNA binding sites (6).

The human TR4 orphan receptor (TR4) was first identified by using degenerate polymerase chain reaction cloning. The open reading frames of TR4 cDNA encode 615 amino acids with a calculated molecular mass of 67 kDa (8). On the basis of sequence similarities, TR4 is classified as a member of the steroid hormone receptor superfamily, very close to the TR2 orphan receptor (9, 10). A comparison of the amino acid sequence in the p-box of the DNA binding domain groups TR4 in the estrogen receptor/thyroid hormone receptor (ER, TR) subfamily, which recognizes the AGGTCA core consensus motif. From this information we were able to identify several target genes that are up-regulated by TR4, including the fifth intron of the ciliary neurotrophic factor receptor (11, 12) and DR4T₃RE (13). In contrast, TR4 represses the SV40 major late promoter (14) and retinoic acid receptor/RXR target genes (15). Similar results were also obtained with TR2 (16, 17). Molecular mechanisms of the differential regulation by TR4 remain unclear. Determining whether TR4 is capable of interfering with gene regulation by binding to other AGGTCA-like motifs and/or interacting with cofactor(s) might provide us more detailed information about this orphan receptor. A vitamin D₃ target gene is a potential candidate of interest for more study, because its receptor functions through interaction with the vitamin D₃ response element, which contains two AGGTCA repeat motifs with a 3-nucleotide space between repeats (DR3-VDRE). These response element motifs may also be recognized by TR4. Despite the similarity of hormone response elements (HRE) recognized by both VDR and TR4, these two receptors have also been found to interact in vitro with the receptor interacting protein 140 (RIP140) cofactor (18, 19). Investigating the regul-

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† The abbreviations used are: RXR, retinoid X receptor; TR4, testicular orphan receptor; HRE, hormone response elements; VDRE, vitamin D response element; GST, glutathione S-transferase; EMISA, electrophoretic mobility shift assay; CHO, Chinese hamster ovary; mAb, monoclonal antibody; CAT, chloramphenicol acetyltransferase; DR, direct repeat; VDR, vitamin D₃ receptor; TR4-RE, thyroid hormone response element; RIP140, receptor interacting protein 140; T₃R, thyroid receptor.

The Orphan Receptor*

In situ hybridization analysis demonstrated that abundant testicular orphan receptor (TR4) transcripts were detected in kidney, intestine, and bone, which are vitamin D₃ target organs. Cell transfection studies also demonstrated that the expression of the vitamin D₃ target gene, 25-hydroxyvitamin D₃ 24-hydroxylase, can be repressed by TR4 through high affinity binding (K_d = 1.32 nM) to the direct repeat 3 vitamin D₃ receptor response element (DR3VDRE). This TR4-mediated repression of DR3VDRE is in contrast to our earlier report that TR4 could induce thyroid hormone target genes containing a direct repeat 4 thyroid hormone response element (DR4T₃RE). Electrophoretic mobility shift assay using several TR4 monoclonal antibodies when combined with either TR4-DR3VDRE or TR4-DR4T₃RE showed a distinct supershifted pattern, and proteolytic analysis further demonstrated distinct digested peptides with either TR4-DR3VDRE or TR4-DR4T₃RE. These results may therefore suggest that TR4 can adapt to different conformations once bound to DR3VDRE or DR4T₃RE. The consequence of these different conformations of TR4-DR3VDRE and TR4-DR4T₃RE may allow each of them to recruit different coregulators. The differential repression of TR4-mediated DR3VDRE and DR4T₃RE transactivation by the receptor interacting protein 140 (RIP140), a TR4 coregulator, further strengthens our hypothesis that the specificity of gene regulation by TR4 can be modulated by protein-DNA and protein-protein interactions.

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lation of TR4 in the vitamin D signaling pathway provides us with a molecular mechanism to explain the way TR4 regulates the gene expression in terms of protein-DNA and protein-protein interactions.

Vitamin D$_3$ is hydroxylated first in the liver at carbon 25 to yield 25-hydroxyvitamin D$_3$ and then in the kidney at the α-position of carbon 1 to generate 1,25-(OH)$_2$D$_3$, the active form of vitamin D. This has various biological effects, including the maintenance of calcium homeostasis, regulation of bone remodeling, and modulation of cell growth and differentiation (20–23). The 25-hydroxymethyl D$_3$ 24-hydroxylase gene (P450cc24) encodes a key enzyme involved in vitamin D metabolism, which is responsible for the conversion of 25-(OH)$_2$D$_3$ and 1,25-(OH)$_2$D$_3$ to 24,25-trihydroxyvitamin D$_3$, respectively (24). These metabolites are thought to be inactive forms of vitamin D (25). However, 1,25-(OH)$_2$D$_3$ induces 24-hydroxylase activity in its target cells, and thus its presence may play a crucial role in eliminating hormone activity of the vitamin D compound (25).

Two vitamin D-responsive elements (VDRE-1 and VDRE-2) responsible for 1,25-(OH)$_2$D$_3$ stimulation of transcription were identified at nucleotides −151 to −137 and nucleotides −225 to −245 of the 5′-flanking region of the rat P450cc24 gene (26–28). Both VDREs contain two AGGTCA-like repeat motifs with a 3-nucleotide space in sense or antisense orientation and are similar to the VDREs found in the human P450cc24 gene (29).

Examination of regulation via the 24-hydroxylase induction mechanism at the molecular level may contribute to the understanding of vitamin D in the endocrine system. In this study, we analyzed the regulation by TR4 on the vitamin D$_3$ signaling pathway and compared the expression pattern of TR4 to that in vitamin D$_3$ target organs. The differential regulation of target genes containing DR3VDRE and DR4T3RE by TR4 was further investigated, and the results suggested that conformational changes because of DNA-protein and protein-protein interactions might play major roles in this regulation.

MATERIALS AND METHODS

Plasmid Construction—For the transient transfection or coupled in vitro transcription/translation of the full-length TR4 protein, the pCMX-TR4 and pET14b-TR4 plasmids were constructed as described previously (14). The chimeric receptor pCMX-A4 was constructed as described (13). The reporter plasmid P450cc24-CAT, the 24-hydroxylase gene, was kindly provided by Dr. Y. Kato (26). The GST fusion protein bound to glutathione-Sepharose-4B beads was incubated for 2 h at 4 °C with 5 μl of in vitro translated TR4 protein (1 μg of each) were incubated with an increasing amount of [35S]methionine-labeled protein in a total volume of 100 μl of incubation buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 5 mM MgCl$_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mg/ml bovine serum albumin, 10% glycerol, and protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin)). The glutathione-Sepharose-4B beads were then washed three times with wash buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mg/ml EDTA, 0.5% Nonidet P-40). The beads were boiled in 2 × SDS sample buffer, loaded onto SDS-polyacrylamide gels, and visualized by autoradiography.

Other Methods—In situ hybridization, electrophoretic mobility shift assay (EMSA), Scatchard analysis, and transient transfection were performed according to the methods described previously (11, 14, 31, 32).

RESULTS

TR4 Specifically Binds to DR3VDRE, the AGGTCA Motif with 3-nucleotide Spacing—EMSA was used to determine the binding specificity of TR4 to DR3VDRE. In vitro translated TR4 protein was incubated with [32P]-labeled DR3VDRE oligomers used in EMSA without or with 1 μl of in vitro translated TR4 (lanes 2–5). A 100-fold excess of unlabeled DR3VDRE (lane 3) was added as a competitor. One microliter of anti-TR4 monoclonal antibodies 4 and 2 were added (lanes 4 and 5). The migration position of the specific binding formed by the DNA-protein complex and the supershifted band formed by adding monoclonal antibodies are indicated with an arrow and an arrowhead, respectively.

TR4 Represses the Vitamin D$_3$-induced Rat P450cc24 Gene Expression—The consequence of high affinity binding between TR4 and DR3VDRE was then investigated by reporter assay.

**FIG. 1. Binding of in vitro expressed TR4 to DR3VDRE.** [32P]-labeled DR3VDRE oligomers were used in EMSA without or with 1 μl of in vitro translated TR4 (lanes 2–5). A 100-fold excess of unlabeled DR3VDRE (lane 3) was added as a competitor. One microliter of anti-TR4 monoclonal antibodies 4 and 2 were added (lanes 4 and 5). The migration position of the specific binding formed by the DNA-protein complex and the supershifted band formed by adding monoclonal antibodies are indicated with an arrow and an arrowhead, respectively.
The target gene used here was the 5'-flanking region (nucleotides 2200 to 1188) of the rat P450cc24, containing VDREs that are responsible for the 1,25-dihydroxyvitamin D3 enhancement and are located at nucleotides 2167 to 2188 and nucleotides 2204 to 2129 (22). Co-transfection of 2.5 mg of pSG5VDR with 3 mg of P450cc24-CAT into CHO cells enhanced the transactivation of P450cc24-CAT to 32-fold in the presence of 10^{-7} M 1,25-(OH)2D3 (Fig. 3B, lane 2 versus 3). However, there is no CAT activity with the co-transfection of 2.5 mg of pCMX-TR4 or pCMX-4A4 either in the absence or presence of 1,25-(OH)2D3 (Fig. 3B, lanes 4–7). The chimeric receptor pCMX-4A4, which replaces the DNA binding domain of TR4 with that of an androgen receptor (Fig. 3A), was unable to bind to the AGGTCA DR motif sequence and served here as a negative control. The transcriptional activity of P450cc24-CAT induced by vitamin D3 was repressed when co-transfected with pCMX-TR4, but not when co-transfected with the chimeric receptor, pCMX-4A4 (Fig. 3B, lanes 3 versus 8 and 9). These results suggest that the DNA binding domain of TR4 is essential for the TR4-mediated repression of the vitamin D3 responsive enhancement of the P450cc24 gene. To examine the expression levels of both wild-type and chimeric receptor TR4 proteins after transfection, polyclonal antibodies against both proteins were produced and examined. This repression effect was further proven by co-transfection of pSG5VDR with different amounts of pCMX-TR4 (from 1 to 5 mg) into CHO cells. As shown in Fig. 4, the repression effect mediated by TR4 was gradually increased when an increasing amount of TR4 was co-transfected (lanes 4–8). This result clearly demonstrated that TR4 could suppress the vitamin D3-induced P450cc24 gene promoter activity in a TR4 dose-dependent manner.

Localization of TR4 Transcripts in Vitamin D3 Target Organs during Mouse Embryogenesis—To determine if TR4 is expressed in vitamin D target tissues, we applied in situ hybridization analysis to mouse embryos. As shown in Fig. 5, high levels of TR4 transcripts were detected in the perichondrium, which contains cells active in bone formation (Fig. 5B). Distinct TR4 distribution was also observed in the developing glomeruli and tubule structures of the kidney as well as the intestinal villi (Fig. 5, C and D). Fig. 5A shows strong TR4 expression detected in certain nonclassical vitamin D target tissues, such as lung and hair follicles (35, 36). Active TR4 expression in these tissues with the known expression domains of VDR (37) and 24-hydroxylase (38, 39) suggests TR4 may interact with the in vivo vitamin D signaling pathway.

The Conformational Differences between TR4-DR3 Complex and TR4-DR4 Complex—The above results suggest that TR4 may exert a repression effect on vitamin D3 responsive target gene expression by binding to DR3Vdre. In our previous studies, we concluded that TR4 activates the expression of the genes that contain DR4T3RE in both HRE sequence- and TR4 dose-dependent manners (13). This contrasting and differential regulation by TR4 could be because of different DNA-protein or protein-protein interactions. When compared, we found the Kd values of TR4 to DR3Vdre and TR4 to DR4T3RE to be very similar (1.32 versus 2.0 nM). This result eliminates the possibility that a different binding capacity between TR4 and DR3 or DR4 results in the distinctive regulation.
The second possibility is that the mechanism of regulation may be through different protein-protein interactions that are dependent on the distinct conformations of TR4 once bound to either DR3VDRE or DR4T3RE. To test this second possibility, EMSA was used to examine a series of monoclonal antibodies raised against TR4, which are able to recognize conformational epitopes. Four monoclonal antibodies (mAbs) were initially raised against TR4, which are able to recognize conformational epitopes. Four monoclonal antibodies (mAbs) were initially characterized by EMSA on the basis of their ability to super-shift or abolish the TR4-DNA complex. As shown in Fig. 6A, we observed a specific DR3/3TR4 complex (lane 2) that was distinct from mock-translated protein (lane 1); with the addition of mAbs 1, 2, and 3, supershifted bands were found (lanes 3–5). In contrast, the specific band formed by DR3/3TR4 was abolished when the mAb 4 was added (lane 6). The same band-shifted pattern was observed when DR3 was replaced with DR4 (Fig. 6B, lane 1–6). Interestingly, different band-shifted patterns were observed with various combinations of different antibodies. As shown in Fig. 6A, an enhanced supershifted band shows migration positions that are different from the supershifted band (lane 8). This suggests that mAbs 1 and 3 recognize different epitopes because their simultaneous addition resulted in an increased mobility shift beyond that of either antibody alone. In contrast, the addition of mAbs 1 and 2 did not lead to an enhanced supershifted band (lanes 3 and 4 versus 7). These results suggest that the mAb 1 recognizes the same epitope of TR4 as the mAb 2 when TR4 is bound to DR3. However, a supershifted band was detected when the mAb 1 was added with the mAb 3 simultaneously to TR4/3DR4 complex (Fig. 6B, lane 8). These results indicate that TR4 might fold into different conformations upon binding to diverse HREs and that different antibodies can recognize these conformations.

This second hypothesis was further proven by proteolytic analysis. [35S]Met/hionine-incorporated, in vitro translated TR4 was incubated in the absence or presence of HRE (DR4 or DR3) at 25°C for 1 h. As shown in Fig. 4C, TR4/3DR4/3RE has a similar trypsin-resistant fragment pattern to that of unbound TR4 control. In contrast, the TR4/3DR3/3RE complex was more sensitive to trypsin when 2.5 μg/ml trypsin was applied. As results showed, the full-length of TR4 was completely degraded and some trypsin-resisting fragments disappeared compared to that with TR4/3DR4/3RE at the same concentration of protease treatment. Similar results were also obtained when we replaced trypsin with α-chymotrypsin. We concluded that TR4-bound DR4/3RE or DR3/3RE had a different sensitivity to protease digestion and different protease-resisting fragments could be obtained with a higher concentration of protease digestion. These results further confirm our hypothesis that, to exert its proper function, TR4 may adopt distinct conformations when bound to DR3VDRE or DR4T3RE, leading to different protein environments.

**RIP140 Interacts with TR4 and Differentially Modulates the TR4-mediated DR3VDRE-CAT and DR4T3RE-CAT Activities**—In vitro interaction of TR4 and RIP140 was performed by GST pull-down assay. As shown in Fig. 7A, TR4 could interact with RIP140 but not RXR (lane 3 versus 4). No interaction could be detected when GST-T3/3RE was replaced with GST (lane 5 and 6). The effects of RIP140 on TR4-mediated DR3VDRE-CAT and DR4T3RE-TE-CAT activities were also investigated. As shown in Fig. 7B, RIP140 can further repress the TR4-mediated DR3VDRE-CAT suppression (lane 8 versus 9). In contrast, RIP140 can repress both TR4 and T3/3RE-mediated DR4T3RE-CAT activities (Fig. 7C, lane 5 versus 6 and lane 7 versus 10) significantly, but RIP140 has no significant effect on the DR4T3RE-TE-CAT induction when TR4 and liganded TR3 were co-transfected (Fig. 7C, lane 8 versus 9). It is also worth noting that whereas RIP140 can further enhance the TR4-mediated DR3VDRE-CAT suppression (lanes 8 versus 9), RIP140 has no significant effect on DR3VDRE-CAT transactivation in the absence of TR4 (Fig. 7B, lane 7 versus 10).

**DISCUSSION**

According to the sequence of the TR4 DNA binding domain and our previous results from EMSA, we conclude that TR4 binds to the AGGTCA core consensus motif arranged in a direct repeat orientation with various numbers of nucleotide spacings. In this paper, we demonstrated that TR4 binds to DR3VDRE with high affinity and thus suppresses vitamin
D₃-induced P450cc24 gene activation. However, in our previous studies, we demonstrated that TR4 activates the genes that contain DR₄·T₃RE or nonclassical T₃RE by binding to their response elements (13). The mechanisms of different binding responses of TR4 to the same sequence in core motifs with different spacings remain unclear. The responsiveness of genes to steroid hormones involves both the binding of regulatory proteins to specific DNA sequences and the formation of critical protein-protein associations. Throughout the past decade, a number of nuclear receptor coregulators has been characterized and provides us a more detailed molecular model of how nuclear receptors regulate their target genes. Because TR4 binds to DR₃·VDRE and DR₄·T₃RE with similar affinities (1.32 versus 2.0 nM), we propose that whether TR4 works as a transcriptional enhancer or a silencer might be mediated not only by direct DNA binding but also by protein-protein interactions. This indicates that in addition to the receptor and the DNA, other factors may also contribute to the selectivity of receptors in the recognition of their target genes.

To test this hypothesis, EMSAs and proteolytic analyses were performed. In Fig. 4, A and B, distinct EMSA patterns were observed when TR4 was bound ³²P·DR₃·VDRE and ³²P·DR₄·T₃RE in the presence of different combinations of various TR4 monoclonal antibodies. Meanwhile, different peptide patterns were obtained when DR₄· or DR₃·bound TR4 was digested with trypsin. Taken together, these data suggest that TR4 binding to diverse HREs may result in distinct conformational changes that can then trigger differential regulation. This finding supports the existence of a unique spacing of the direct repeat, which serves as a binding site for an auxiliary protein that modifies receptor activity (39). Similar approaches have also been used to study a thyroid receptor response element, RSV·T₃RE, which contains an inverted repeat with a 6-nucleotide space. RSV·T₃RE allows strong activation by c-ErbA in the absence of thyroid hormone, and the results of antibody-induced supershift experiments indicate that binding to this element may result in a different conformation as compared with binding to a typical DR₄·T₃RE (40). These results suggest that different conformational changes may be involved in determining whether TR4 would function as a positive or negative regulatory factor.

As previous studies indicated that RIP140 could interact with VDR, T₃R, and TR4 (18, 41), we were interested in determining if RIP140 could differentially affect TR4-mediated gene induction and repression. Using the GST pull-down assay, we demonstrated that TR4 could interact in vitro with RIP140. We then proved that RIP140 could also enhance the trans-repressive effect of TR4 on the vitamin D₃-signaling pathway. In contrast, RIP140 has only marginal effects on the vitamin D₃-induced VDRE-CAT activity, although it might interact with VDR (18). On the other hand, RIP140 repressed the trans-activation mediated by both TR4 (Fig. 7B, lane 5 versus 6) and liganded T₃R (Fig. 7B, lane 7 versus 10) on DR₄·T₃RE-CAT. However, RIP140 had only very marginal repressive effects on DR₄·T₃RE-CAT once we overexpressed TR4 and liganded T₃R simultaneously. A reasonable explanation for the effect of
RIP140 on DR4-T3RE-CAT is that TR4 and liganded T3R may both interact with RIP140 and sequester RIP140 blocking the repressive effect of RIP140 on DR4-T3RE-CAT (Fig. 8). Because RIP140 can repress both pathways mediated by TR4, it might not be recruited differently by TR4 when it is bound to DR4-T3RE or DR3-VDRE. However, RIP140 did show differential effects in the regulation of DR4-T3RE-CAT and DR3-VDRE-CAT when receptors, TR4 and VDR versus TR4 and T3R, are co-transfected. These data support the role of the protein environment surrounding the DNA as a key factor in determining gene regulation. Therefore, the dynamic interaction between the receptors and cofactors in response to their target DNA may determine the specificity of gene regulation.

Although the functional reporter assay presented here was carried out in vitro, the expression patterns of TR4 in the vitamin D target organs support the potential role of TR4 in regulation of the in vivo vitamin D pathway in the endocrine system. Vitamin D and its receptor play essential roles in the regulation of calcium homeostasis and bone formation. Bone, kidney, and intestine are three major targets for such action. In addition, vitamin D is involved in the regulation of cell proliferation, differentiation, and the immune response. At present, the nonclassic targets reported include lung (35) and hair follicles (36). It has been demonstrated that high levels of VDR
the binding affinity of TR4 and RXR/VDR to DR3VDRE and found that TR4 bound to DR3VDRE with a 5.5-fold higher affinity than the RXR/VDR heterodimer. These binding affinity data suggest that TR4 may be able to compete with RXR/VDR to bind to DR3VDRE and in this way, exert its repressive effect. The ratio of functional TR4-RXR3VDRE complex to RXR/VDR/DR3VDRE might also be important in determining target gene action. Additionally, the failure of the repression effect observed when PCMX-TR4 was replaced with the chimeric receptor PCMX-4A4 in a reporter gene assay further supports the idea that DNA binding is essential for TR4 to exert its proper function.

The responsiveness of genes to a steroid hormone receptor is principally mediated by functional interactions between DNA-bound receptors and components of the transcription initiation machinery. In this paper we demonstrated that TR4 represses the P450cc24 gene activation induced by vitamin D₃ via in vitro and in vivo evidence. Both DNA binding and proper protein-protein interactions may be the key factors in determining the specific function of TR4. This study may lead to an understanding of the role of DNA binding in altering the conformation of TR4 and allowing different protein interactions resulting in a complex that is capable of mediating differential regulation.

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