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# A Mutant Thyroid Hormone Receptor $\beta$1 Identified in a Patient with Resistance to Thyroid Hormone Inhibits the Activities of Not Only the Wild-Type TRs, but also Other Nuclear Receptors

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**Although mutations of human thyroid hormone receptor $\beta$ (hTR$\beta$) have been associated with resistance to thyroid hormone (RTH), the molecular basis by which the mutant TRs cause the various clinical symptoms is unknown. We show here that a mutant TRE $\beta$ identified in a patient with RTH inhibited the transcriptional activities of, not only the wild-type TR$\beta$, but also other nuclear receptors including retinoid X receptor$\alpha$ (RXR$\alpha$), vitamin D3 receptor (VDR) and retinoic acid receptor (RAR$\alpha$). We provide evidence that these inhibitions by the mutant TRE$\beta$ occur by different mechanisms. Namely, the mutant TR$\beta$ interferes with VDR and RAR$\alpha$ by competition for binding to the corresponding response elements, but the pathway through RXR$\alpha$ is mainly inhibited by squelching of RXR$\alpha$ in solution. These findings suggest that in patients with RTH, not only the T3 responsive genes but also other responsive genes are inhibited by the mutant TRs, which might explain the variety of clinical symptoms in RTH.** 

The nuclear hormone receptors are ligand-dependent transcriptional factors that mediate various aspects of growth, development, and homeostasis by binding as homodimers or heterodimers to their cognate DNA elements. The retinoid-X receptor (RXR), vitamin D3 receptor (VDR), thyroid hormone receptor (TR) and retinoic acid receptor (RAR) activate transcription by binding to DNA-response elements that consist of direct repeats spaced by 1, 3, 4 and 5 base pairs, respectively(1, 2, 3).

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Resistance to thyroid hormone (RTH) is characterized by poor responsiveness of peripheral or pituitary tissues to thyroid hormone (T3) and an inappropriate TSH secretion in spite of a high level of T3. This syndrome is caused by various mutations in the hormone binding domain of the T3 receptor (TR)$\beta$1(4, 5, 6). Many mutant TR$\beta$1s responsible for RTH interfere with the actions of both TR$\alpha$ and $\beta$(7, 8). However, little is known about the mechanisms explaining the clinical features, such as mental retardation and short statue. We(9) have found a mutant TR$\beta$1 with a truncation of 11 carboxyl(C)-terminal amino acids (F451X, designated according to the nomenclature of TR$\beta$ gene mutation in the consensus statement from the first workshop on RTH(10)), in a 6-year-old Japanese girl with severe RTH. This subject had impairment of the central nervous system. F451X was shown to have strong dominant negative effects on wild-type TR$\beta$.

In the present study, we examined the influence of F451X on the transcriptional activities of nuclear receptors including RXR$\alpha$, VDR and RAR$\alpha$.

## MATERIALS AND METHODS

*A patient.* Clinical features and laboratory data on this patient have already been reported(9).

*Plasmid construction.* Human TR$\beta$1cDNA (pCMX-hTR$\beta$1), human RXR$\alpha$ (pCMX-hRXR$\alpha$), human VDR (pCMX-VDR), human RAR$\alpha$ (pCMX-RAR$\alpha$) and reporter genes (DR1-, DR3-, DR5-CAT) were kindly provided by Dr. Evans (Howard Hughes Medical Institute, The Salk Institute for Biological Studies, San Diego, CA, USA) and Dr. Umesono (Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-01, JAPAN). The M13mp18 replicative form, which contained the patient's TR$\beta$1 gene, was digested with Bgl II and Taq I, and the DNA fragment of about 100 bp containing the mutations was recovered. This fragment was replaced with the identical position of the wild-type TR$\beta$1 cDNA. The mutations and the correct linkages between the restriction enzyme sites of the expression vector (pCMX-F451X) was confirmed by sequencing DNA



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of the constructed plasmid. The expression plasmid F-LBD containing only the ligand binding domain was made by digesting F451X with Tth111 I and Kpn I, removing about a 750 bp fragment. After the treatment with T4 DNA polymerase to be blunt ends, the plasmid was ligated.

*Cell culture and transient transfection assay.* CV-1 cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with kanamycin (80 $\mu$g/ml) and 10% fetal bovine serum(FCS) in 60 mm plates at 37 °C in a humidified incubator with 5 % $CO_2$ in air. Cells at a density of $10^6$ cells/plate were transfected with 0.36 $\mu$ g of the expression vector containing RXR$\alpha$, VDR or RAR$\alpha$ and mutant TRE $\beta$ together with 1.8 $\mu$g reporter plasmid of DR1-, DR3- or DR5-CAT by the calcium phosphate method. After exposure to calcium phosphate-DNA precipitates for 20 h, the cells were washed with PBS and incubated for a further 24 h in DME medium containing 10% FCS with indicated ligands (9cis-retinoic acid (RA), vitamin D3 or all-trans-RA). The cells were harvested and CAT enzyme activities were measured, as described previously(9). To normalize transfection efficiency, $\beta$-galactosidase was included as an internal control. Results represent the mean of three or more experiments.

*Gel mobility shift assay (EMSA).* The DR1-DNA (sense strand: 5′-TCAGCTTCAGGTCAGAGGTCATCGAC-3′, anti-sense strand: 5′-TCAGGTCGATGACCTCTGACCTGAAG-3′) fragment, DR3-DNA (sense strand: 5′-AGCTATAGGTCAAGGAGGTCAGCGATCT-3′, anti-sense strand: 5′-CAGTAGATCGCTGACCTCCTTGACCTAT-3′) and DR5-DNA (sense strand: 5′-TCAGCTTCAGGTCACCAGGAG-GTCATCGAG-3′, anti-sense strand: 5′-TGCACTCGATGACCTCCT-GGTGACCTGAAG-3′) were synthesized. The annealed probe was labeled by the protocol of digoxigenin (DIG) Gel Shift Kit (Boehringer Mannheim, Mannheim, Germany). Each receptor was *in vitro* transcribed and translated using T7 RNA polymerase II and rabbit reticulocyte lysate according to the manufacturer's instruction (PICA-MAC, Toyo Inki, Tokyo, Japan). The translated VDR or RAR $\alpha$ (2$\mu$l) with or without RXR $\alpha$ (2 $\mu$l), or RXR $\alpha$ (4 $\mu$l) were incubated with 1 $\mu$l of DIG-labeled oligonucleotides (0.5 pmol/$\mu$l) in a reaction mixture (20 $\mu$l) containing 10 mM Tris-HCl (pH 8.0), 40 mM KCl, 0.05 % Nonidet P-40 (NP-40), 0.2 $\mu$g poly(dI-dC), 6% glycerol, 4 mg/ml bovine serum albumin (BSA), 1 mM DTT and at room temperature for 10 min. The mixtures were loaded on 4% polyacrylamide gel containing 4 % glycerol in 0.5×TBE running buffer (0.045 M Tris-borate, 0.001 M EDTA) at 10 V/cm at room temperature. Oligonucleotides were transferred onto nylon membrane. DNA-protein complexes were detected by a chemiluminescent method according to the manufacturer's procedure (Boehringer Mannheim, Mannheim, Germany).

## RESULTS

*Inhibition of 9 cis-RA/RXR $\alpha$-dependent regulation by F451X.* The inhibitory effect of F451X on the transcriptional activity of RXR$\alpha$ was examined by co-transfecting RXR$\alpha$ and F451X into CV-1 cells at an equal molar ratio. The CAT activity was assayed using the reporter gene of DR1-CAT(Fig. 1). Compared with the cells transfected with RXR$\alpha$ alone, the cells transfected with RXR$\alpha$ and F451X showed significantly reduced CAT activities. Wild-type TR$\beta$ also inhibited the activity of RXR$\alpha$, although to a lesser extent, irrespective of the presence or absence of T3 (data not shown). Interestingly, the activity of RXR$\alpha$ was significantly inhibited by co-transfection of increasing amounts of F-LBD which had only the ligand binding domain of F451X (Fig.1).

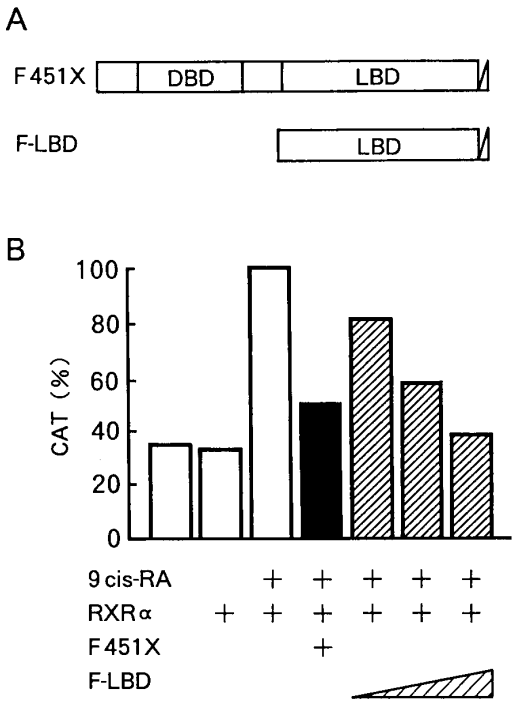


**FIG. 1.** Inhibition of 9 cis-RA-RXR$\alpha$-dependent activation by F451X. (**A**) Schematic representation of the mutant TR$\beta$(F451X) and F-LBD. F451X is truncated 11 carboxyl-terminal amino acids of TR$\beta$1($\Delta$). F-LBD contains only the ligand binding domain (LBD) of F451X. (**B**) CV-1 cells were transfected with or without RXR$\alpha$ (360 ng) and in the absence or presence of F451X (360 ng) or increasing amounts of F-LBD (180, 360 and 720 ng), together with the DR1-CAT reporter plasmid (1.8$\mu$g). Each sample contains 7.2 $\mu$g DNA with the additional mock DNA. After 24h treatment with 1 $\mu$M 9 cis-RA or vehicle, the cells were harvested and CAT activities were measured. The data were normalized to the CAT activity of RXR$\alpha$ at 1 $\mu$M 9 cis-RA. Four independent experiments were performed. Although the standard deviations were not indicated, they were smaller than 12 %.

*Inhibition of vitamin D3/VDR- or RA/RAR $\alpha$-dependent regulation by F451X.* We also studied whether F451X affected the VDR or RAR$\alpha$ function. By co-transfection of increasing amounts of F451X into CV-1 cells, the ligand dependent transcriptional activities of VDR (Fig. 2A) and RAR$\alpha$ (Fig. 2B) were significantly inhibited. The activities of VDR and RAR$\alpha$ were not inhibited by increasing amounts of F-LBD (Fig. 2C). Although wild-type TR$\beta$ also inhibited the activities of VDR and RAR$\alpha$ in the absence of T3, this inhibition was eliminated when more than 100 nM T3 was present (data not shown).

*F451X inhibits the activities of several nuclear receptors by distinct mechanisms.* Whether F451X interferes with the receptor binding to DNA was examined by gel shift experiments. The strong RXR $\alpha$ homodimer band was observed on DR1-RXRE only when 9-cis RA was present (Fig. 3A, compare lane 1

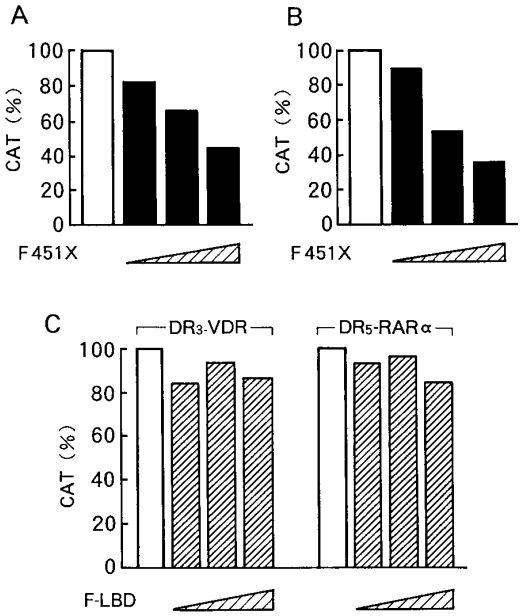


**FIG. 2.** Inhibition of 1, 25 vitamin D3 (100 nM VtD3)-, all-trans retinoic acid (100 nM RA)-dependent activations by F451X. (**A, B**) CV-1 cells were transfected with RAR$\alpha$ or VDR in the absence or presence of F451X (360 ng), together with the DR3-CAT or DR5-CAT reporter plasmid (1.8$\mu$g), respectively. Each CAT activity is relatively expressed as that of VDR at 100 nM VtD3(**A**), or that of RAR$\alpha$ at 100 nM RA(**B**). Four independent experiments were performed and the results of a representative experiment are shown. The standard deviations were less than 10 %. (**C**) CV-1 cells were transfected with VDR (100 nM VtD3) or RAR$\alpha$ (100nM RA) in the absence or presence of increasing amounts of F-LBD (180, 360 and 720 ng), together with the DR3- or DR5-CAT reporter plasmid (1.8 $\mu$g), respectively. Each CAT activity is relatively expressed as that of VDR at 100 nM VtD3 (left bars), or that of RAR$\alpha$ at 100 nM RA (right bars). Each sample contains the same amount of DNA (7.2 $\mu$g). Four independent experiments were performed and the results of a representative experiment are shown. The standard deviations were less than 12 %.

and lane 3). Although the F451X homodimer band was not observed on DR1-RXRE (data not shown), the faint RXR$\alpha$/F451X heterodimer band appeared in the absence of 9-cis RA (Fig. 3A, compare lane 3 with lane 7). This heterodimer band was supershifted by an anti-TR antiserum (Fig. 3A, lane 8). Interestingly, the addition of increasing amounts of F-LBD lead to a clear reduction of RXR $\alpha$ homodimer band (Fig. 3A, lane 4-6). Taken together with the findings shown in Fig. 1, these findings suggest that F451X sequestrated RXR$\alpha$ by forming RXR$\alpha$/F451X heterodimer complex in solution, diminishing RXR$\alpha$ availability for binding to DR1-DNA. RXR$\alpha$/RAR$\alpha$ heterodimer binding to DR5-RARE was, on the other hand, not reduced by increasing amounts of F-LBD and RXR$\alpha$/F451X heterodimer band appeared (Fig. 3B). The findings obtained with VDR were similar to those obtained with RAR$\alpha$ (data not shown).

## DISCUSSION

Each nuclear hormone receptor had been believed to function autonomously in gene regulation, activating or repressing target gene transcription independently in a highly hormone-specific fashion. However, it has recently understood that the nuclear hormone receptors are implicated in the receptor network and that the activity of one receptor can be influenced by other members of the receptor family (11-13). Consequently, the mutations in these receptors might generate transdominant effects and influence the network of signaling.

The patients with resistance to thyroid hormone (RTH) have one mutant and three (one TR$\beta$ and two TR$\alpha$) normal alleles and the product of the mutant allele inhibits normal TR function in a dominant negative manner (4, 14). We studied whether a mutant TR$\beta$ influences the actions of other nuclear receptors. In cotransfection studies, a mutant TR$\beta$1(F451X) interfered with the activities of, not only wild-type TR$\beta$ but also other nuclear receptors (RXR$\alpha$, VDR and RAR$\alpha$). Interestingly, the activity of RXR$\alpha$ was also inhibited by F-LBD, which only contained the ligand binding domain of F451X. In EMSA, the binding of F451X to DR1-DNA was weak and F-LBD inhibited the RXR$\alpha$ homodimer band efficiently. On the other hand, F-LBD did not inhibit the activity of VDR or RAR $\alpha$. In addition,

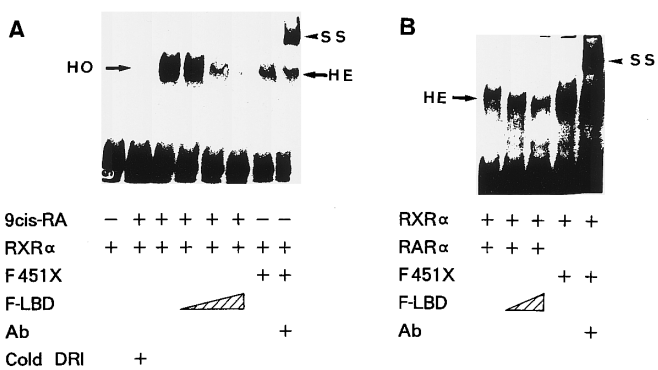


**FIG. 3.** F451X inhibits the actions of RXR$\alpha$, VDR and RAR$\alpha$ by different mechanisms. (**A**) Each lane contains 1 $\mu$l of DIG-labeled DR1-oligonucleotides and *in vitro*-translated RXR$\alpha$ with or without increasing amounts of *in vitro*-translated FLBD (2, 4 and 6 $\mu$l) or F451X(2 $\mu$l) in the absence or presence of 1 $\mu$M 9 cis-RA. Reticulocyte lysate was added to some samples so that the total volume of reticulocyte lysate was the same for each sample. Note that one sample was treated with the anti-TR$\beta$ antibody for 15 min at room temperature before EMSA (lane 8). HO, RXR$\alpha$ homodimer; HE, F451X-RXR$\alpha$ heterodimer; ss, supershifted band. (**B**) Each lane contains 1 $\mu$l of DIG-labeled DR5-oligonucleotides and *in vitro*-translated RAR$\alpha$ and RXR$\alpha$ with or without increasing amounts of *in vitro*-translated F-LBD (2, 4 and 6 $\mu$l) or F451X(2 $\mu$l) in the presence of 100 nM RA. Note that one sample was treated with the anti-TR$\beta$ antibody for 15 min at room temperature before EMSA (lane 5). HE, RXR$\alpha$-RAR$\alpha$ heterodimer or RXR$\alpha$-F451X heterodimer; ss, supershifted band.

F451X bound DR5-DNA and DR3-DNA(data not shown), and the RXR$\alpha$-RAR$\alpha$ heterodimer band and RXR$\alpha$-VDR heterodimer band (data not shown) were not attenuated by F-LBD. These findings suggest differences in the mechanisms by which nuclear receptors are inhibited.

The cross-talk of nuclear hormone receptor-related pathways have been shown at several levels of the signal transduction cascade: (I) at the level of hormone response elements (HREs), such as the competition for binding to common response elements (15-17); (II) at the level of protein-protein interactions of receptors with other transcription factors (18-20); (I) at the level of the competition for co-factors (including RXR) for the activation of receptors (squelching) (21-25); (IV) at the level of the direct active silencing to the transcriptional machinery (26); or (V) at the level of hormone binding as glucocorticoids that bind to GR and MR (27). Our findings suggest that F451X inhibits the action of RXR$\alpha$ by squelching (III), but the activities of VDR and RAR$\alpha$ are inhibited through by DNA competition (I). Interestingly, these findings are similar to those reported by Privalsky et al. (28), that v-erbA oncoprotein inhibits the activities of RAR$\alpha$ and RXR$\alpha$ and that v-erbA binding to DNA is essential for suppression of RAR$\alpha$ but that v-erbA repression of RXR function occurs through a different mechanism independently of DNA recognition.

F451X we used is a C-terminal truncated TR$\beta$ and structurely resembles v-erbA. F451X was shown to exhibit the potent dominant negative effects on wild-type TR$\beta$, just like v-erbA. The patient with F451X has been shown to have severe clinical symptoms, including mental retardation and attention deficit hyperactivity disorder (ADHD) (9). Although the molecular mechanisms by which the mutant TRs cause these clinical symptoms are unknown, the potent dominant negative mutant might inhibit the complex of the signal transduction pathways by various nuclear receptors, which might be responsible for the severe clinical signs of this patient with RTH.

Transcriptional silencing may also contribute to the physiological disturbances associated with RTH. Unliganded TR functions as a transcriptional repressor of genes bearing thyroid hormone response elements in their promoters. Interestingly, the silencing activity of F451X was significantly greater than that of the wild-type TR$\beta$. The basal transcription level on TREpal2 under the T3-free condition was decreased to 77.4 $\pm$ 26.3 % by wild-type TR$\beta$ and 26.7 $\pm$ 7.0 % by F451X ($p < 0.05$, n=4 experiments) (9). Baniahmad et al. demonstrated that two different RTH mutants functioned as constitutive repressors of target genes with strong silencing activity(29). These findings suggest that the dominant negative RTH mutant inhibiting several receptors, also retains an active and constitutive silencing function. In this respect, co-repressors which have recently been cloned (30-32) might be associated with these phenomena and they would be very useful as additional tools to understand the roles of TRs in the transcriptional repression.

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