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ELSEVIER

Association of Tumor Necrosis Factor and Human Leukocyte Antigen DRB1 Alleles with Graves' Ophthalmopathy

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ABSTRACT: Tumor necrosis factor (TNF)-α plays a central role in the development of ophthalmopathy in patients with Graves' disease (GD). The aim of this study was to investigate the association of TNF promoter polymorphisms at positions -1031 (T-1031C), -863 (C-863A), -857 (C-857T), -308 (G-308A), and -238 (G-238A) with Graves' ophthalmopathy (GO). We studied the distribution of TNF and human leukocyte antigen (HLA) DRB1 alleles in 228 Polish white patients with GD, 106 of whom had ophthalmopathy (NOSPECS class ≥III) and 248 healthy subjects. TNF -308A and HLA-DRB1*03 alleles were significantly increased in patients with GD compared with healthy subjects. Stratification analysis revealed no independent association of -308A with GD when the DRB1*03 status was considered. Subdividing GD according to eye involvement revealed that the distribution of TNF promoter haplotypes differed significantly in patients with or without ophthalmopathy. The haplotype containing the -238A allele was absent in GO. The association of G-238A with GO was independent of DRB1 alleles. These results indicate that TNF G-308A is associated with susceptibility to GD (however, this association is not independent of HLA-DRB1*03) and that TNF G-238A is associated with the development of ophthalmopathy, suggesting that G-238A or a gene in linkage disequilibrium may be disease modifying in GD. *Human Immunology 65, 632–639 (2004).* © American Society for Histocompatibility and Immunogenetics, 2004. Published by Elsevier Inc.

KEYWORDS: genetic polymorphisms; Graves' disease; HLA; ophthalmopathy; TNF

ABBREVIATIONS

GD	Graves' disease
GO	Graves' ophthalmopathy
HLA	human leukocyte antigen
SNP	single nucleotide polymorphism
TNF	tumor necrosis factor

INTRODUCTION

Graves' ophthalmopathy (GO) is an autoimmune inflammatory disorder of the extraocular muscles and the orbital fat/connective tissue that is closely associated with Graves' hyperthyroidism. Cytokines are likely to play an important role in the initiation and propagation of the autoimmune process in the orbit [1]. The proinflammatory reactions mediated by tumor necrosis factor (TNF)-α include an induction of expression of adhesion molecules on endothelial cells [2]. TNF-α influences also the expression of a potentially important autoantigen (thyrotropin receptor) and certain immunomodulatory proteins (human leukocyte antigen [HLA] DR, ICAM-1, heat shock protein 72) on orbital fibroblasts, which are considered to be the target cell of the autoimmune attack [1, 3].

High levels of TNF-α were found in retrobulbar tissues samples in GO, and the enlargement of extraoc-

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ular muscles was significantly correlated with TNF mRNA expression [4, 5]. Increased serum levels of TNF-α have been also reported in patients with active GO [6, 7]. Because the production of TNF-α has been demonstrated to be under genetic control, TNF gene may be considered as an important candidate gene contributing to the development and/or severity of GO [8]. In our previous study, we have demonstrated that TNF single nucleotide polymorphisms (SNPs) at positions -1031 (T→C change, termed T-1031C) and -863 (C-863A) were associated with ophthalmopathy in Japanese patients with Graves' disease (GD) [9]. In the present study, we have investigated the associations of five SNPs located in the 5′ promoter/enhancer region at positions -1031 (T-1031C), -863 (C-863A), -857 (C-857T), -308 (G-308A), and -238 (G-238A) with GO in a Polish white population. In addition, we analyzed the frequencies of HLA-DRB1 alleles in patients with GD with and without ophthalmopathy because of the strong linkage disequilibrium within the HLA region.

MATERIALS AND METHODS

Subjects

We studied a total of 228 randomly selected Polish patients with GD recruited from the Department of Endocrinology, Medical University of Warsaw, and 248 healthy Polish adults recruited from the Blood Transfusion Center. The diagnosis of GD was based on the presence of hyperthyroidism, diffuse goiter, detectable thyroid-stimulating hormone receptor autoantibodies (TRAK Lumitest, BRAHMS Diagnostica, Germany) and/or increased radioiodine uptake. Patients with GD were subdivided into two groups, GO or GD without GO, according to the presence of clinically evident ophthalmopathy, as previously described [10].

Group 1: GO. The GO group comprised 106 patients (81 women, 25 men) aged 14–77 years (median, 47 years). The severity of eye changes was assessed according to the NOSPECS classification. Patients with proptosis (NOSCPECS class III), extraocular-muscle dysfunction (class IV), exposure keratitis (class V), and optic neuropathy (class VI) were considered clinically evident. Patients were categorized according to their highest ever NOSPECS class (class III, 56 patients; class IV, 41; class V, 3; and class VI, 6). The mean duration between the onset of GD and assessment for the study was 4.0 ± 6.0 years.

Group 2: GD without clinically evident ophthalmopathy. The GD without clinically evident ophthalmopathy group (NOSPECS class 0-II) comprised 122 patients (99 women, 23 men) aged 16–78 years (median, 40 years). The research program was approved by the local ethical committee, and all subjects provided written informed consent for genetic studies.

TNF Gene Polymorphism Analysis

Polymorphisms in the TNF promoter (GenBank Accession number: L11698) were identified by dot-blot hybridization with sequence-specific oligonucleotide probes, as previously described [9, 11].

HLA-DRB1 Genotyping

HLA-DRB1 typing was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP) with the Dynal All Set SSP DR test (Dynal Biotech, Bromborough, Wirral, UK). HLA-DRB1 alleles were identified in all patients with GD and in 125 healthy subjects, who have been previously reported [12].

Statistical Analysis

The frequencies of TNF genotypes and HLA-DRB1 carriers were compared between groups by χ^2 test or Fisher's exact probability test with a 2 × 2 contingency table. Bonferoni's correction for multiple testing was applied. A corrected p (p_c) value of <0.05 was considered significant. Odds ratios (ORs) were calculated according to Woolf's method. When one element in the 2 × 2 table was zero, the OR was calculated with the formula modified by Haldane: $OR = \{[(2a + 1)(2d + 1)]/[(2b + 1)(2c + 1)]\}$ [13]. TNF haplotypes were estimated from population genotype data by PHASE version 2.02 software [14, 15]. The differences in estimated haplotype frequencies were analyzed by a case-control permutation test with 1000 iterations implemented in the PHASE software package. The Hardy-Weinberg equilibrium test was performed by Arlequin software version 2.000 (Genetics and Biometry Lab, Department of Anthropology, University of Geneva).

RESULTS

Association of TNF and HLA-DRB1 Alleles With GD

In all studied groups, the distribution of TNF genotypes was consistent with the Hardy-Weinberg equilibrium. There were significant differences in TNF G-308A genotype distributions, with an excess of A/A and A/G genotypes in patients with GD compared with healthy controls ($p = 0.0004$, $p_c = 0.006$, OR = 2.0) (Table 1). There was a tendency toward a decrease in TNF -238 A/A and A/G genotypes in GD ($p = 0.018$, $p_c = 0.27$, OR = 0.4). There were no significant differences in genotype frequencies of any TNF gene polymorphism between male and female patients with GD (data not shown).

TABLE 1 Genotype frequencies of TNF promoter polymorphisms in healthy subjects and in patients with Graves' disease (GD) with or without ophthalmopathy[a]

TNF polymorphism	Genotype	Healthy subjects (n = 248)	GD total (n = 228)	GD without ophthalmopathy (n = 122)	GD with ophthalmopathy (n = 106)
T-1031C	C/C	5 (2.0%)	5 (2,2%)	4 (3.3%)	1 (0.9%)
	T/C	76 (30.6%)	69 (30.3%)	39 (32.0%)	30 (28.3%)
	T/T	167 (67.3%)	154 (67.5%)	79 (64.8%)	75 (70.8%)
C-863A	A/A	5 (2.0%)	2 (0.9%)	1 (0.8%)	1 (0.9%)
	C/A	53 (21.4%)	63 (27.6%)	33 (27.0%)	30 (28.3%)
	C/C	190 (76.6%)	163 (71.5%)	88 (72.1%)	75 (70.8%)
C-857T	T/T	9 (3.6%)	2 (0.9%)	2 (1.6%)	0 (0%)
	C/T	64 (25.8%)	61 (26.8%)	34 (27.9%)	27 (25.5%)
	C/C	175 (70.6%)	165 (72.4%)	86 (70.5%)	79 (74.5%)
G-308A	A/A	4 (1.6%)	10 (4.4%)	5 (4.1%)	5 (4.7%)
	G/A	72 (29.0%)	96 (42.1%)	52 (42.6%)	44 (41.5%)
	G/G	172 (69.4%)	122 (53.5%)[b]	65 (53.3%)	57 (53.8%)
G-238A	A/A	1 (0.4%)	0 (0%)	0 (0%)	0 (0%)
	G/A	22 (8.9%)	8 (3.5%)	8 (6.6%)	0 (0%)
	G/G	225 (90.7%)	220 (96.5%)[c]	114 (93.4%)	106 (100%)[d,e]

[a] p values were calculated with the use of χ^2 test or Fisher's exact probability test and corrected (p_c) for the number of tests performed (n = 15).

OR, odds ratio. GD versus healthy subjects: [b] $p = 0.0004$, $p_c = 0.006$, OR = 2.0; [c] $p = 0.018$, $p_c = 0.27$, OR = 0.4; GD with ophthalmopathy vs. GD without ophthalmopathy: [d] $p = 0.008$, $p_c = 0.12$, OR = 0.1; GD with ophthalmopathy versus healthy subjects: [e] $p = 0.0003$, $p_c = 0.0045$, OR = 0.05.

The distribution of HLA-DRB1 carriers is listed in Table 2. A strong association for GD was seen with the DRB1*03 allele ($p = 0.00001$, $p_c = 0.0004$, OR = 2.9). Because TNF G-308A is known to be in strong linkage disequilibrium with the A*01-Cw*0701-B*0801-DRB1*0301-DQB1*0201 haplotype, we stratified subjects according to the DRB1*03 and -308A status (Table 3) [16]. Only the frequency of DRB1*03(+)/TNF-308A(+) carriers was significantly increased in GD compared with healthy subjects, suggesting that any association between TNF G-308A and susceptibility to GD resulted from linkage disequilibrium with HLA-DRB1*03.

Association of TNF and HLA-DRB1 Alleles With GO

Subdividing patients with GD according to clinical eye involvement revealed that the -238A allele was absent in

TABLE 2 HLA-DRB1 carrier frequencies in healthy subjects and in patients with Graves' disease (GD) with or without ophthalmopathy[a]

HLA-DRB1	Healthy subjects (n = 125)	GD total (n = 228)	GD without ophthalmopathy (n = 122)	GD with ophthalmopathy (n = 106)
01	22 (17.6%)	30 (13.2%)	16 (13.1%)	14 (13.2%)
15	28 (22.4%)	60 (26.3%)	30 (24.6%)	30 (28.3%)
16	6 (4.8%)	20 (8.8%)	9 (7.4%)	11 (10.4%)
03	26 (20.8%)	98 (43.0%)[b]	52 (42.6%)	46 (43.4%)
04	27 (21.6%)	36 (15.8%)	16 (13.1%)	20 (18.9%)
11	30 (24.0%)	62 (27.2%)	33 (27.0%)	29 (27.4%)
12	12 (9.6%)	8 (3.5%)[c]	5 (4.1%)	3 (2.8%)
13	38 (30.4%)	55 (24.1%)	35 (28.7%)	20 (18.9%)[d]
14	6 (4.8%)	12 (5.3%)	4 (3.3%)	8 (7.5%)
07	27 (21.6%)	37 (16.2%)	23 (18.9%)	14 (13.2%)
08	8 (6.4%)	17 (7.5%)	10 (8.2%)	7 (6.6%)
09	2 (1.6%)	3 (1.3%)	1 (0.8%)	2 (1.9%)
10	1 (0.8%)	3 (1.3%)	2 (1.6%)	1 (0.9%)

[a] p values were calculated with the use of χ^2 test (with Yates' correction where appropriate) or Fisher's exact probability test and corrected (p_c) for the number of tests performed (n = 39).

OR, odds ratio. GD versus healthy subjects: [b] $p = 0.00001$, $p_c = 0.0004$, OR = 2.9; [c] $p = 0.033$, $p_c = 1.0$, OR = 0.3; GD with ophthalmopathy versus healthy subjects: [d] $p = 0.044$, $p_c = 1.0$, OR = 0.5.

TABLE 3 Distribution of HLA-DRB1*03 and TNF-308A carriers in healthy subjects and patients with Graves' disease (GD) with or without ophthalmopathy[a]

Carriers		Healthy subjects	GD total		GD without ophthalmopathy	GD with ophthalmopathy	
HLA-DRB1*03	TNF-308A	(n = 125)	(n = 228)	OR1	(n = 122)	(n = 106)	OR2
−	−	79 (63.2%)	108 (47.4%)	0.5[b]	56 (45.9%)	52 (49.1%)	1.1
+	−	5 (4.0%)	14 (6.1%)	1.6	9 (7.4%)	5 (4.7%)	0.6
−	+	20 (16.0%)	22 (9.7%)	0.6	14 (11.5%)	8 (7.5%)	0.6
+	+	21 (16.8%)	84 (36.8%)	2.9[c]	43 (35.2%)	41 (38.7%)	1.2

[a] Odds ratios (OR) were calculated according to Woolf's method, comparing GD total versus healthy subjects (OR1) and GD with ophthalmopathy versus GD without ophthalmopathy (OR2). p values were calculated by χ^2 test or Fisher's exact probability test; [b] $p = 0.004$; [c] $p = 0.0001$.

patients with ophthalmopathy, and only -238G/G homozygotes were detected (GO vs. GD without ophthalmopathy: $p = 0.008$, $p_c = 0.12$, OR = 0.1; GO vs. healthy subjects: $p = 0.0003$, $p_c = 0.0045$, OR = 0.05) (Table 1). We have previously reported that cigarette smoking and age at onset of GD of >42 years were associated with the development of GO [10]. However, T-1031C, G-868A, C-857T, and G-308A genotype frequencies remained insignificantly different between patients with GD with or without ophthalmopathy, even after stratification for cigarette smoking status, sex, and age at onset of GD (data not shown). There was also no significant association between the frequency of -1031C, -868A, -857T, and -308A alleles and the severity of eye changes in patients with GD: NOSPECS class 0-I (20%, 14%, 14%, and 25%, respectively), class II (19%, 19%, 23%, and 27%), class III (19%, 19%, 13%, and 26%), and class IV–VI (11%, 11%, 13%, and 25%).

On the basis of the genotype data, we reconstructed TNF promoter haplotypes (Table 4). In all studied groups, the genetic variation in the TNF promoter could be explained by five or six haplotypes, which together accounted for >99% of all haplotypes. The frequencies of individual haplotypes in healthy subjects were similar to those reported recently by Zeggini *et al.* [17, 18]. The distribution of TNF haplotypes differed significantly between patients with GD with or without ophthalmopathy ($p = 0.002$). The haplotype containing -1031C and -238A alleles and the haplotype containing only the -1031C allele were absent in patients with GO. The later haplotype was also absent in healthy controls and thus may be regarded as "specific" for Graves' hyperthyroidism without ophthalmopathy.

The distribution of HLA-DRB1 carriers did not differ significantly in patients with GD with or without ophthalmopathy (Table 2). In patients with ophthalmopathy, the frequency of HLA-DRB1*03(+)/TNF-308A(+) carriers was significantly increased compared with healthy subjects ($p = 0.0002$). However, the distribution of DRB1*03 and -308A carriers did not differ significantly between patients with GD with and without ophthalmopathy (Table 3). The frequency of a potentially protective HLA-DRB1*07 allele, which has been previously associated with ophthalmopathy [19–21], tended to be lower in patients with GO compared with healthy controls ($p = 0.08$, OR = 0.5) (Table 2). Because TNF -238A allele has been reported to be in linkage disequilibrium with HLA-DRB1*07, we performed

TABLE 4 Estimated TNF promoter haplotype frequencies in healthy subjects and in patients with Graves' disease (GD) with or without ophthalmopathy[a]

TNF promoter haplotype					Healthy subjects	GD without ophthalmopathy	GD with ophthalmopathy		
−1031	−863	−857	−308	−238	(n = 248)	(n = 122)	(n = 106)	OR1	OR2
T	C	C	G	G	50.5%	39.8%	46.7%	0.9	1.3
T	C	T	G	G	16.3%	15.6%	12.7%	0.7	0.8
T	C	C	A	G	15.6%	25.4%	25.5%	1.9	1.0
C	A	C	G	G	12.0%	14.3%	15.1%	1.3	1.1
C	C	C	G	A	4.6%	3.3%	0%	0.05	0.07
C	C	C	G	G	0%	1.6%	0%	1.0	0.1
				Sum	99%	100%	100%		

[a] Boldface indicates the less common allele. Only haplotypes which occurred with a frequency >1% in at least one studied group are shown. Odds ratios (OR) were calculated according to Woolf's method (with Haldane's modification where appropriate), comparing GD with ophthalmopathy versus healthy subjects (OR1) and GD with ophthalmopathy versus GD without ophthalmopathy (OR2). p values were estimated by a case-control permutation test, comparing the distribution of TNF haplotypes in GD with ophthalmopathy versus healthy subjects ($p1 = 0.004$) and GD with ophthalmopathy versus GD without ophthalmopathy ($p2 = 0.002$).

TABLE 5 Distribution of HLA-DRB1*07 and TNF-238A carriers in healthy subjects and patients with Graves' disease (GD) with or without ophthalmopathy[a]

Carriers		Healthy subjects (n = 125)	GD without ophthalmopathy (n = 122)	GD with ophthalmopathy (n = 106)	OR1	OR2
HLA-DRB1*07	TNF-238A					
–	–	92 (73.6%)	94 (77.0%)	92 (86.8%)	2.4[b]	2.0
+	–	23 (18.4%)	20 (16.4%)	14 (13.2%)	0.7	0.8
–	+	6 (4.8%)	5 (4.1%)	0 (0%)	0.1[c]	0.1[d]
+	+	4 (3.2%)	3 (2.5%)	0 (0%)	0.1	0.2

[a] Odds ratios (OR) were calculated according to Woolf's method (with Haldane's modification where appropriate), comparing GD with ophthalmopathy versus healthy subjects (OR1) and GD with ophthalmopathy versus GD without ophthalmopathy (OR2). p values were calculated by χ^2 test or Fisher's exact probability test: [b] $p = 0.01$; [c] $p = 0.02$; [d] $p = 0.04$.

stratification analysis in order to examine whether the association of TNF -238A with ophthalmopathy was dependent of HLA-DRB1*07 (Table 5). Although frequencies of both DRB1*07(+) and -238A(+) carriers were decreased in ophthalmopathy compared with healthy subjects and GD without ophthalmopathy, the differences were significant only with DRB1*07(-)/-238A(+) individuals. In addition, after removing patients with GD carrying the HLA-DRB1*07 allele, the distribution of TNF promoter haplotypes remained significantly different between patients with or without GO ($p = 0.012$, data not shown). These results suggest that the "protective effect" of the -238A allele was independent of DRB1*07.

DISCUSSION

GD is a heterogeneous autoimmune disorder affecting the thyroid, eyes, and skin with varying degrees of severity [22]. Although characteristic changes in retrobulbar tissues are detectable on orbital imaging in almost all patients with GD, clinically apparent ophthalmopathy occurs in 30%–50% of patients, with severe and potentially sight-threatening forms affecting 3%–5% of patients. The reason for this variation in the clinical presentation of eye changes is unclear. Given the pathophysiological role of TNF-α, we hypothesized that TNF gene may be an important candidate gene contributing to the development and/or severity of GO. Therefore, we studied TNF promoter SNPs at positions -1031, -863, -857, -308, and -238 with respect to the susceptibility to GD and ophthalmopathy.

In patients with GD, the frequency of TNF -308A/A and A/G genotypes were significantly increased compared with healthy controls. The association of G-308A with GD, as well as with a variety of infectious and autoimmune diseases, has been already reported [23–27]. However, the functional significance of the G-308A polymorphism remains controversial [16, 28, 29]. In addition, investigations of the role of TNF G-308A are complicated by the strong linkage disequilibrium with the HLA haplotype A*01-B*0801-DRB1*0301-DQA1*0501-DQB1*0201, which is known to confer susceptibility to various autoimmune diseases [16]. In order to assess the independent role of TNF G-308A polymorphism, we performed HLA-DRB1 typing, which confirmed the known association between DRB1*03 and GD [30]. The OR conferred by DRB1*03 allele (OR = 2.9) was similar to previously reported in European white populations (2.5–4.3). The DRB1*03 allele appeared to be more strongly associated with GD than the TNF -308A allele (OR = 1.9). In addition, the stratification analysis suggested that -308A allele did not confer susceptibility to GD independently of DRB1*03, which is in accordance with the study by Hunt and colleagues [23].

Although linkage and/or association studies implicate the HLA region in susceptibility to GD, the role of HLA in modifying the disease phenotype remains unclear [30]. Recently, TNF haplotypes have been reported to be associated with specific manifestations of ulcerative colitis, scleroderma, and asthma [31–33]. Subdividing patients with GD according to the presence of clinically evident eye diseases revealed that the distribution of TNF promoter haplotypes differed significantly between patients with or without ophthalmopathy. The -238A/-1031C haplotype was absent in GO, whereas the haplotype containing only the -1031C allele was "specific" for Graves' hyperthyroidism without eye disease. Moreover, TNF -238A/A and A/G genotypes were absent in GO. The TNF-238A allele is not known to be of functional significance but is located close to a putative repressor site [34, 35]. TNF G-238A polymorphism has been associated with susceptibility to or severity of various autoimmune diseases [17, 35–39]. Similarly to our results, TNF -238A/A and A/G genotypes have been reported to be absent in patients with severe rheumatoid arthritis and were associated with a lower rate of joint damage [35, 36, 39]. Thus, G-238A or a gene in linkage disequilibrium may have an disease-modifying effect in

rheumatoid arthritis and GD. Nevertheless, the clinical relevance of the G-238A polymorphism is limited because the -238A allele is rare in white populations (~5%), and it represents only a small contribution to overall susceptibility to autoimmune diseases.

The results of HLA-DRB1 associations with GO are contradictory [30]. In white patients with GO, the frequency of HLA-DRB1*03 allele has been reported to be increased, decreased, or unchanged, compared with GD without eye disease [21, 40–42]. Our data revealed a nearly identical distribution of HLA-DRB1*03 and TNF -308A alleles in patients with GD with or without ophthalmopathy, suggesting that DRB1*03 does not influence the development of eye disease in patients with GD. The HLA-DRB1*07 is a protective allele for patients with GD [23, 43]. Reduced frequencies of HLA-DRB1*07 have been reported especially in juvenile GD and in some studies in GO [21, 44, 45]. In our study, the frequency of DRB1*07 carriers tended to be lower in GO compared with healthy subjects ($p = 0.08$). Although the TNF -238A allele has been reported to be in linkage disequilibrium with HLA-DRB1*07, the stratification analyses performed suggest that the association of G-238A with GO was independent of DRB1*07 [19, 20].

In our previous study, we have reported that TNF -1031C and -863A alleles were associated ophthalmopathy in Japanese patients with GD [9]. There was no relationship between G-308A and G-238A SNP's and susceptibility to GD or GO in the Japanese population. However, the distribution of TNF alleles differed significantly in the two populations, with -308A and -238A alleles being significantly higher in whites. Therefore, this contradictory results may reflect different genetic susceptibility to GD and GO in different ethnic groups [46]. Nevertheless, both studies suggest that TNF may play a role in the development of ophthalmopathy and further studies are required to detect "true" susceptibility or protective genes within the HLA region.

In conclusion, the results our study suggest that TNF G-308A is associated with susceptibility to GD (however, this association is not independent of that seen for HLA-DRB1*03); and that TNF G-238A is associated with the development of ophthalmopathy, which is independent of HLA-DRB1*07. Although our study suggests that TNF G-238A, or a gene in linkage disequilibrium, may have an disease-modifying effect in GD, a further study of sufficient power is needed to confirm this observation.

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Vitamin D Receptor As an Intestinal Bile Acid Sensor

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The vitamin D receptor (VDR) mediates the effects of the calcemic hormone 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. We show that VDR also functions as a receptor for the secondary bile acid lithocholic acid (LCA), which is hepatotoxic and a potential enteric carcinogen. VDR is an order of magnitude more sensitive to LCA and its metabolites than are other nuclear receptors. Activation of VDR by LCA or vitamin D induced expression in vivo of CYP3A, a cytochrome P450 enzyme that detoxifies LCA in the liver and intestine. These studies offer a mechanism that may explain the proposed protective effects of vitamin D and its receptor against colon cancer.

A contributing factor to the deleterious effects of a high-fat diet is an associated increase in the excretion of fecal bile acids (*1*), the most toxic of which is the secondary bile acid LCA (Fig. 1A). Unlike the primary bile acids, chenodeoxycholic acid (CDCA) and cholic acid (CA), LCA is poorly reabsorbed into enterohepatic circulation and passes into the colon. At high concentrations, LCA induces DNA strand breaks, forms DNA adducts, and inhibits DNA repair enzymes (*1–3*). LCA can also promote colon cancer in animals (*4*), and its concentration is higher than other secondary bile acids in patients with colorectal cancer (*5*).

In contrast to the positive correlation among high-fat diets, LCA, and colon cancer, dietary intake of vitamin D and calcium is related to a reduced incidence of colorectal cancer (*6*). Furthermore, vitamin D supplementation inhibits colon carcinogenesis induced by either high-fat diets or intrarectal instillation of LCA (*7*, *8*). One route for LCA elimination is through its catabolism by the enterohepatic cytochrome P450, CYP3A, a putative target gene of vitamin D (*9*, *10*). Expression of CYP3A in the liver is regulated by the nuclear xenobiotic and pregnane X receptor (PXR, also called SXR), which can be activated by high concentrations (≥100 μM) of LCA (*11*, *12*). Primary bile acids (in particular, CDCA and CA) are also ligands for the farnesoid X receptor, FXR (*13*, *14*). However, neither PXR nor FXR responds to vitamin D, and LCA-induced expression of CYP3A is still present in PXR-null animals. This suggests another LCA-dependent pathway for inducing CYP3A expression (*11*).

To determine if bile acids could act on the vitamin D receptor (VDR) to induce CYP3A expression, we used a ligand-screening assay based on the ligand-induced interaction of a nuclear receptor with its coactivator (*14*). The receptor-interacting domain of the coactivator SRC-1 was fused to the DNA binding domain of the yeast transcription factor GAL4, and various nuclear receptors were fused to the transactivation domain of the herpes virus VP16 protein. Expression plasmids for GAL4-SRC-1 and VP16-nuclear receptor were transfected with a GAL4-responsive luciferase reporter plasmid into human embryonic kidney (HEK293) cells and examined for luciferase expression after LCA treatment. LCA (30 μM) induced a ligand-dependent interaction between VDR and SRC-1 (Fig. 1B). As previously reported (*14*), LCA also activated FXR. However, no other nuclear receptors were activated by LCA (Fig. 1B) (*15*), including PXR, which required higher LCA concentrations (≥100 μM) to be activated. To further investigate the ligand specificity of VDR and FXR, we tested various primary, secondary, and conjugated bile acids in this assay (Fig. 1C). We performed these experiments in the presence or absence of the ileal bile acid transporter (IBAT), because hydrophilic bile acids such as CA and conjugated bile acids require transport across cell membranes (*16*). As expected, treatment of cells with the vitamin D hormone 1,25$(OH)_2D_3$ activated VDR but not FXR (Fig. 1C). Conversely, the primary bile acids CDCA, CA, and their conjugated metabolites were effective ligands for FXR but not VDR. FXR was also activated by the secondary bile acids, deoxycholic acid, LCA, and their conjugated metabolites (Fig. 1C). However, the only bile acids that activated VDR were LCA and its major metabolites 3-keto-LCA (Fig. 1A), glyco-LCA, and 6-ke-

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to-LCA (Fig. 1C, shaded area). The 6-keto metabolite of LCA is of further interest because it did not activate FXR. Hence, although VDR and FXR both serve as bile acid receptors, they have distinct specificity profiles.

We also compared the dosage dependency of bile acids to activate human VDR, FXR, and PXR. LCA and 3-keto-LCA activated human VDR with median effective concentration (EC_{50}) values of 8 μM and 3 μM, respectively (Fig. 1D). These bile acids also activated FXR, but effective concentrations were 2 to 3 times greater than those for VDR (Fig. 1E). Although CDCA was an FXR agonist (EC_{50} = 7 μM), it was not effective on VDR at any concentration. LCA and 3-keto-LCA were also equally effective at activating full-length mouse or human VDR [see below and (*15*)]. The xenobiotic receptor PXR also is activated by LCA (*11*, *12*). However, compared to VDR, PXR required a concentration at least 10 times greater than that of either LCA (Fig. 1F) or 3-keto-LCA (*15*) for activation. Therefore, the concentration at which these bile acids activate VDR is below the pharmacologic range that activates FXR and PXR. These data suggest that VDR is a more sensitive receptor for bile acids than are FXR and PXR, specifically for LCA and its major metabolite 3-keto-LCA.

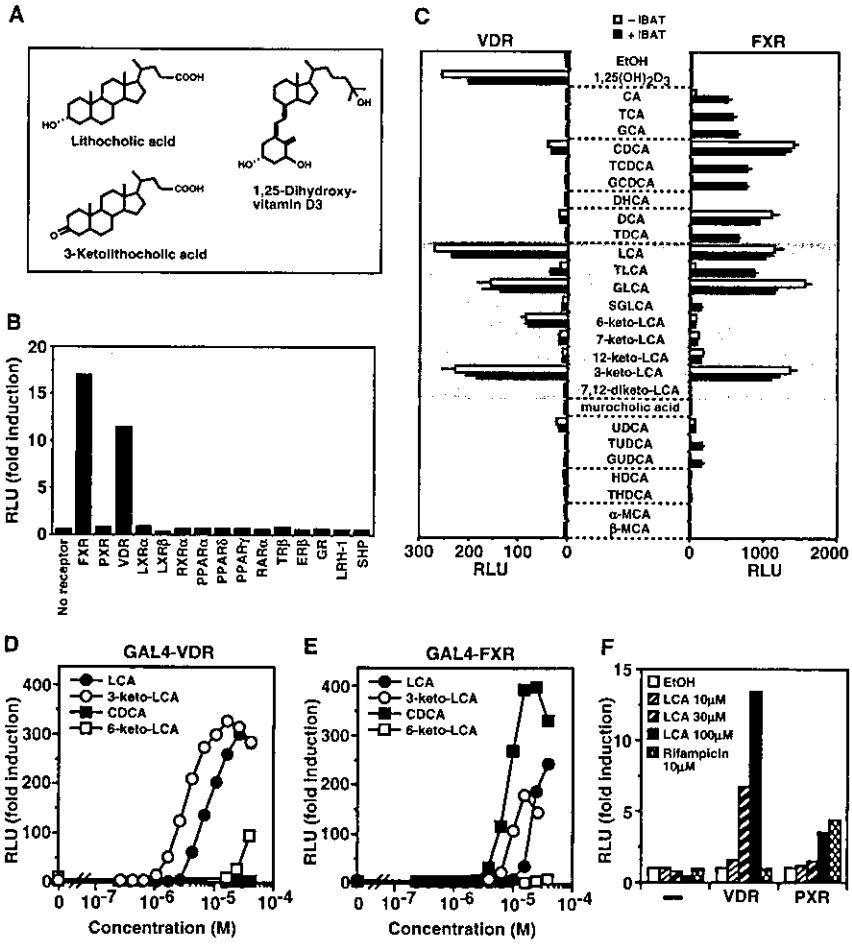

Fig. 1. LCA and its metabolites are VDR agonists. (**A**) Structures of VDR agonists. (**B**) Receptor-specific activation by LCA. Various nuclear receptors were expressed in HEK293 cells and screened for activation by 30 μM LCA with a mammalian two-hybrid GAL4-SRC-1 and VP16-receptor luciferase assay. (**C**) Ligand specificity of VDR- and FXR-activation by bile acids. VDR and FXR were screened for activation by $1,25(OH)_2D_3$ (0.1 μM) or various bile acids (30 μM), as in Fig. 1B. The screen was performed on transfected HEK293 cells expressing VDR or FXR in the presence (black bars) or absence (white bars) of IBAT to facilitate uptake of CA and the conjugated bile acids (*16*). The shaded area identifies LCA-specific metabolites. (**D** and **E**) Comparative dose response of human VDR and FXR to bile acids using a GAL4-receptor luciferase assay. (**F**) Comparative dose response of VDR and PXR to bile acids. Transfection assay was as in (D) and (E), except that full-length human VDR and PXR were expressed in monkey kidney CV1 cells with a luciferase reporter plasmid containing three copies of the human CYP3A4 ER6 element (see Fig. 3). Rifampicin was used as a positive control ligand for PXR. RLU, relative light units. Fold inductions by various ligands in (B), (D), (E), and (F) are relative to ethanol (EtOH) vehicle used as a control. See supporting online material for additional methods and bile acid abbreviation.

To demonstrate that bile acids directly bind VDR as ligands, we performed a competitive binding assay using $[^3H]1,25(OH)_2D_3$ and increasing concentrations of candidate bile acids (Fig. 2). Both LCA and 3-keto-LCA competed effectively with $[^3H]1,25(OH)_2D_3$ for binding to VDR [inhibition constant (K_i) = 29 ± 6 μM and 8 ± 3 μM, respectively], with 3-keto-LCA exhibiting an affinity that is 3.5 times greater than that of LCA. A fluorescence polarization assay also showed that LCA, but not other bile acids, induced association with the coactivator peptide (fig. S1).

LCA is catabolized by CYP3A (*11*, *17*), a putative target gene of vitamin D in the intestine (*9*, *10*). To explore the role of LCA and VDR in the activation of CYP3A, we investigated the promoters of the mouse, rat, and human CYP3A genes for potential VDR-RXR heterodimer binding sites (Fig. 3A). All three gene promoters have direct repeats separated by three nucleotides (DR3 elements) similar to those found in other VDR target genes (*18*). In addition, the human CYP3A4 gene contains an everted repeat separated by six nucleotides (ER6) that has also been reported to be a VDR-RXR response element (*10*). Each of these elements bound to the VDR-RXR heterodimer (Fig. 3B), competed for the receptor heterodimer with high affinity (Fig. 3C), and was responsive to VDR-dependent transactivation by either LCA or $1,25(OH)_2D_3$ (Fig. 3D). It is noteworthy that these elements were previously reported to mediate the xenobiotic response of the PXR-RXR heterodimer in the liver (*19*). In a comparison to PXR, we found that all three CYP3A promoters were at least 10 times more sensitive to VDR when activated by LCA (*15*). The human CYP3A elements were the most sensitive and conferred the strongest

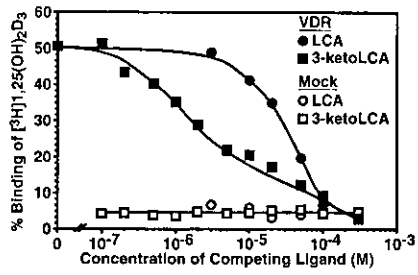

Fig. 2. LCA and 3-keto-LCA directly bind VDR in vitro. Human VDR was expressed in monkey kidney COS-7 cells, labeled with $[^3H]1,25(OH)_2D_3$, and used for competitive binding assays. Results obtained with VDR-containing lysates (closed symbols) or mock lysates transfected with RXR (open symbols) are shown. Data points are representative of three independent experiments using LCA and 3-keto-LCA as competitors. Similar experiments using CDCA, CA, muricholic acid (MCA), and hyodeoxycholic acid (HDCA) showed no competitive binding. See supporting online material for materials and methods.

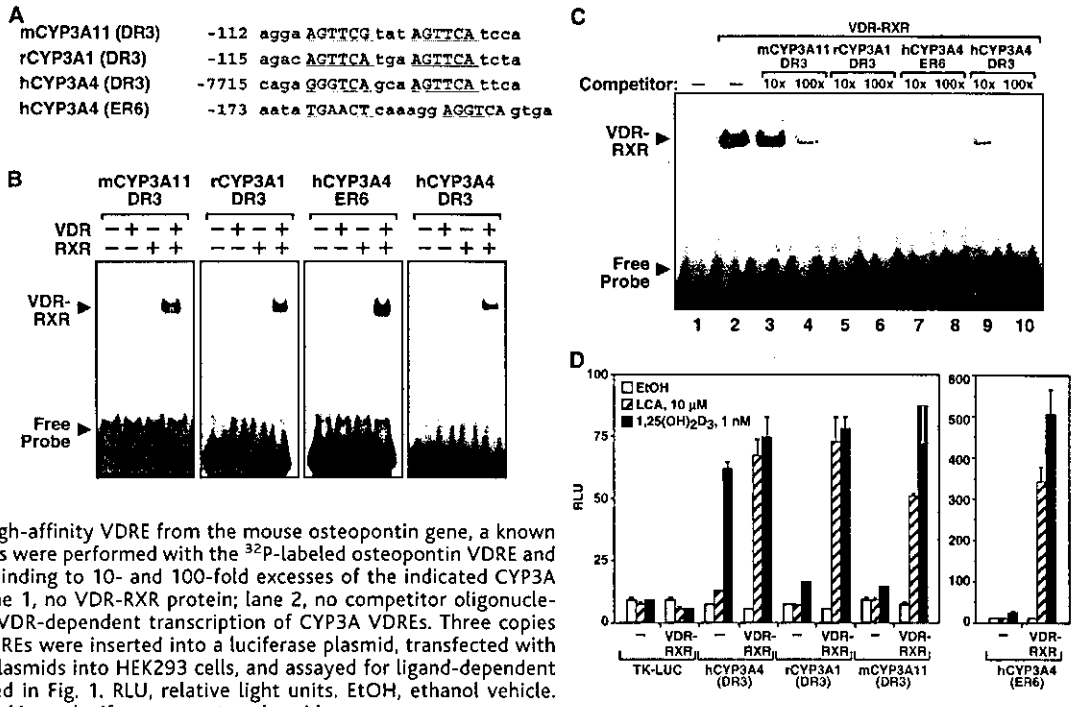

Fig. 3. CYP3A genes are LCA-dependent VDR target genes. (**A**) VDR response element (VDRE) sequences from mouse (m), rat (r), and human (h) CYP3A genes. The numbers indicate positions in the gene promoter relative to the transcription start site. (**B**) VDR-RXR heterodimers bind to CYP3A VDREs. In vitro synthesized RXR and VDR were used in electrophoretic mobility band shift assays (*24*) with [^{32}P]-oligonucleotides shown in (A). The arrowheads depict DNA bound VDR-RXR and free probe. (**C**) CYP3A VDREs compete for VDR-RXR binding to the high-affinity VDRE from the mouse osteopontin gene, a known VDR target (*18*). Band shifts were performed with the ^{32}P-labeled osteopontin VDRE and analyzed for competitive binding to 10- and 100-fold excesses of the indicated CYP3A VDREs (lanes 3 to 10). Lane 1, no VDR-RXR protein; lane 2, no competitor oligonucleotide. (**D**) LCA stimulates VDR-dependent transcription of CYP3A VDREs. Three copies of the indicated CYP3A VDREs were inserted into a luciferase plasmid, transfected with VDR and RXR expression plasmids into HEK293 cells, and assayed for ligand-dependent transactivation as described in Fig. 1. RLU, relative light units. EtOH, ethanol vehicle. TK-LUC, control thymidine kinase luciferase reporter plasmid.

response (Fig. 3D). These data further suggest that VDR mediates LCA-dependent induction of CYP3A gene expression.

To confirm the effects of VDR on induction of CYP3A expression in vivo, the transactivation of the CYP3A11 gene was determined after treating mice with agonists for VDR, FXR, or PXR. For these experiments, we used 1α-hydroxyvitamin D_3 (1α(OH)D_3) (*8*) and EB1089 (*20*) as synthetic VDR agonists, pregnenolone-16α-carbonitrile (PCN) as a PXR-selective agonist (*19*), and LCA as a panagonist for all three receptors. CYP3A11 mRNA expression in the intestine was increased in response to both VDR- and PXR-specific ligands, as well to LCA. In contrast, the VDR-specific target gene calbindin 9K (*18*) was activated by LCA and the VDR-selective agonists 1α(OH)D_3 and EB1089, but not by PCN, indicating that LCA can function as a VDR agonist in vivo. Likewise, the FXR target gene, ileal bile acid binding protein (*14*), was transactivated by LCA, but not by VDR or PXR selective ligands. None of the compounds altered the expression of VDR (Fig. 4A). To demonstrate that LCA- and VDR-dependent activation in vivo does not require PXR, the expression of CYP3A11 was examined in $PXR^{-/-}$ mice and $PXR^{+/-}$ control mice (Fig. 4, B and C). As expected (*11*, *12*), CYP3A11 mRNA expression in response to PCN was eliminated in the liver and intestine of PXR-null mice. However, CYP3A11 mRNA expression was still induced by both the VDR-selective ligands and LCA. This demonstrates that VDR can function as an LCA sensor in vivo, resulting in increased expression of CYP3A.

Taken together, these results point to VDR as a potential bile acid sensor in the enteric tract, where elevated concentrations of LCA may bind to VDR. This "adopted orphan" function of VDR (*21*) complements its endocrine role in the small intestine as a high-affinity [dissociation constant (K_d) = 0.1 to 1 nM] receptor for 1,25(OH)$_2$$D_3$ to promote calcium and phosphate absorption, which ensures proper mineralization of bone (*18*). The regulation of the LCA/VDR metabolic cascade is strikingly similar to that mediated by other evolutionarily related nuclear receptors [e.g., PXR, constitutive androstane receptor (CAR), FXR, and liver X receptor (LXR)] that function as lipid sensors and mediate detoxification of their ligands (*21*). By binding to VDR, both LCA and vitamin D may activate a feed-forward catabolic pathway that increases CYP3A expression and leads to the detoxification of LCA. These findings suggest a model to explain how the enteric system could protect itself from the potentially harmful effects of LCA and why vitamin D is protective against colon cancer under normal physiologic conditions. Protection provided by VDR activation may become compromised when the detoxification pathway is overwhelmed (e.g., by increased levels of LCA due to sustained high-fat diets) or under clinical conditions of vitamin D deficiency (e.g., rickets/osteomalacia). Consistent with this model, there is an epidemiologic relation between the incidence of colon cancer and Western-style, high-fat diets (*22*), and the highest death rates from colon cancer occur in areas with a high prevalence of rickets (*6*). Furthermore, mice lacking VDR not only have rickets but also display enhanced cellular proliferation in the colon (*23*). Thus, this work should provide the impetus for further studies addressing the role of diet, bile acids, and vitamin D in colorectal cancer.

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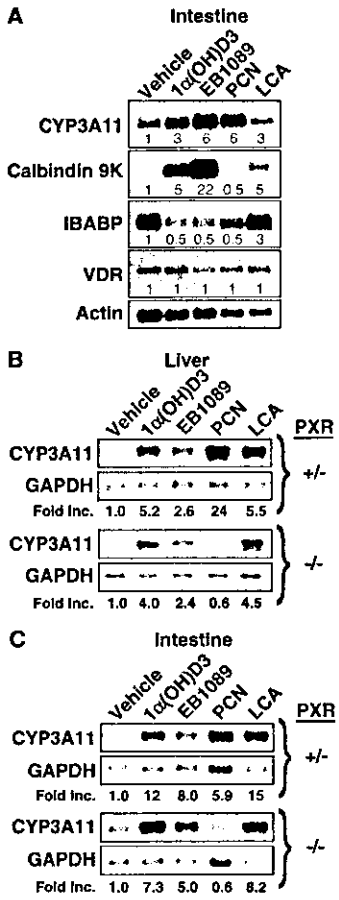

Fig. 4. LCA activation of VDR target genes in vivo. (**A**) Regulation of VDR, PXR, and FXR target gene expression in small intestine. Mice were treated with the indicated ligands or vehicle (corn oil) for 3 days, and small intestine was harvested for Northern blot (RNA) analysis (*25*) using 5 μg pooled mRNA (n = 6 animals). (**B** and **C**) Regulation of CYP3A11 expression in liver and intestine of $PXR^{-/-}$ and $PXR^{+/-}$ mice. Northern blot analysis was performed on liver of mice treated as in (A). Representatives of three independent experiments are shown. The numbers under the lanes indicate fold increases in expression relative to the vehicle and were standardized against actin or glyceraldehyde phosphate dehydrogenase (GAPDH) controls. 1αD3,1α-hydroxy-vitamin D_3.

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25. Male mice (129Sv wild type, $PXR^{+/-}$, or $PXR^{-/-}$) were gavaged daily with vehicle (corn oil), 1.5 μg $1\alpha(OH)D_3$ (gift from M. Pechet, Research Institute for Medicine and Chemistry, Cambridge, MA), 1.5 μg EB1089 (gift from L. Binderup, Leo Pharmaceutical Products, Copenhagen), 1.5 mg PCN, or 8 mg LCA. Mice were killed, and mRNA from the intestine and liver were isolated for Northern blot analysis (*24*) using the indicated gene-specific cDNA probes.

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Structural Determinants for Vitamin D Receptor Response to Endocrine and Xenobiotic Signals

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The vitamin D receptor (VDR), initially identified as a nuclear receptor for 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], regulates calcium metabolism, cellular proliferation and differentiation, immune responses, and other physiological processes. Recently, secondary bile acids such as lithocholic acid (LCA) were identified as endogenous VDR agonists. To identify structural determinants required for VDR activation by 1α,25$(OH)_2D_3$ and LCA, we generated VDR mutants predicted to modulate ligand response based on sequence homology to pregnane X receptor, another bile acid-responsive nuclear receptor. In both vitamin D response element activation and mammalian two-hybrid assays, we found that VDR-S278V is activated by 1α,25$(OH)_2D_3$ but not by LCA, whereas VDR-S237M can respond to LCA but not to 1α,25$(OH)_2D_3$. Competitive ligand binding analysis reveals that LCA, but not 1α,25$(OH)_2D_3$, effectively binds to VDR-S237M and both 1α,25$(OH)_2D_3$ and LCA bind to VDR-S278V. We propose a docking model for LCA binding to VDR that is supported by mutagenesis data. Comparative analysis of the VDR-LCA and VDR-1α,25$(OH)_2D_3$ structure-activity relationships should be useful in the development of bile acid-derived synthetic VDR ligands that selectively target VDR function in cancer and immune disorders without inducing adverse hypercalcemic effects. (*Molecular Endocrinology* 18: 43–52, 2004)

THE VITAMIN D RECEPTOR [VDR (NR1I1)] is a member of the nuclear receptor (NR) superfamily of ligand-inducible transcription factors that regulate many physiological processes including cell growth and differentiation, embryonic development, and metabolic homeostasis (1). The transcriptional activity of NRs is modulated by ligands such as steroids, retinoids, and other lipid-soluble compounds. Upon ligand binding, NRs undergo a conformational change that results in the dissociation of corepressors and recruitment of coactivators (2). These cofactors form complexes with associated factors that induce chromatin remodeling or recruitment of the basal transcription machinery. These interactions allow NRs to modulate transcription of specific target genes.

The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], regulates calcium metabolism, cellular differentiation, and immunity through VDR activation (3, 4). Vitamin D is a secosteroid, in which the B ring of steroid structure is ruptured (5). UV irradiation induces a photochemical reaction of 7-dehydrocholesterol, which is synthesized from acetyl-coenzyme A and is a precursor of cholesterol, to produce the secosteroid vitamin D_3 in the skin. Vitamin D_3 is hydroxylated at the 25 position by vitamin D_3 25-hydroxylase (CYP27A1) in the liver to yield 25-hydroxyvitamin D_3, the major form of vitamin D in the circulation. The 25-hydroxyvitamin D_3 is further hydroxylated in the 1α position by CYP27B1. This reaction is tightly regulated and occurs exclusively in the kidney to yield the active metabolite, 1α,25$(OH)_2D_3$. Three-dimensional modeling and the solution of a crystal structure have yielded valuable insight into the mode of binding of 1α,25$(OH)_2D_3$ in the VDR ligand-binding pocket (LBP) (6–8).

Synthetic vitamin D analogs have been used successfully in the treatment of bone and skin disorders. However, their adverse effects, including hypercalcemia, bone resorption, and soft tissue calcification, limit the clinical application of VDR agonists in the management of malignant tumors and immune disorders (3). The need for VDR ligands with potent anticancer activity that lack adverse effects on calcium metabolism has led to a major synthetic chemistry effort. Several analogs exhibit efficient antiproliferation and

Abbreviations: CAR, Constitutive androstane receptor; FXR, farnesoid X receptor; GST, glutathione-*S*-transferase; h, human; HEK, human embryonic kidney; LBD, ligand binding domain; LBP, ligand binding pocket; LCA, lithocholic acid; NR, nuclear receptor; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PXR, pregnane X receptor; SRC, steroid receptor coactivator; VDR, vitamin D receptor; VDRE, vitamin D response element.

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prodifferentiation activities with fewer calcemic side effects than $1\alpha,25(OH)_2D_3$, but the underlying molecular mechanism of this functional specificity is still not understood (3). Structure-function analysis of vitamin D analogs suggests that these secosteroids also act on a membrane receptor and that adverse effects are at least partly due to a poorly characterized nongenomic mechanism of action (9). To develop efficient VDR-targeting therapy, it is very important to elucidate the structure-function relationship of VDR and its ligands.

Recently, we discovered that VDR functions as a receptor for a number of secondary bile acids including lithocholic acid (LCA) (10). LCA is also a weak agonist for the nuclear receptors farnesoid X receptor (NR1H4) and pregnane X receptor [PXR, also called SXR (NR1I2)]. The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized from cholesterol in liver and secreted in bile as glycine or taurine conjugates (11). After assisting in the digestion and intestinal absorption of lipids and fat-soluble vitamins, including dietary vitamin D, the majority of bile acids are reabsorbed and returned to the liver through the enterohepatic circulation. Bile acids that escape reabsorption in the ileum are converted to the secondary bile acids deoxycholic acid and LCA by intestinal microflora. Whereas farnesoid X receptor serves as a sensor for both primary and secondary bile acids (12–14), PXR and VDR are selectively activated by secondary bile acids (10, 15, 16). PXR, which shares the most sequence identity with VDR, responds to steroid hormone metabolites and xenobiotics, but not to $1\alpha,25(OH)_2D_3$ (17, 18). The crystal structures of the VDR and PXR ligand binding domains (LBDs) reveal strong structural conservation (8, 19). In this study, we compared the LBD structures of VDR and PXR to generate VDR point mutants that selectively respond to $1\alpha,25(OH)_2D_3$ or LCA. Computational docking analysis was used to model the structural requirement for the mutated residues in ligand discrimination. Identification of critical residues for response to endocrine and bile acid ligands should aid the development of VDR agonists with improved pharmacological specificity.

RESULTS

Comparison of LBDs of VDR and PXR

Because human (h) VDR and hPXR share significant amino acid identity (44% in the LBDs) and PXR is responsive to LCA but not $1\alpha,25(OH)_2D_3$, we hypothesized that substitution of VDR residues with PXR amino acids at critical LBP positions that hydrogen bond with the hydroxyl groups of $1\alpha,25(OH)_2D_3$ might yield LCA-selective mutants. The VDR-$1\alpha,25(OH)_2D_3$ cocrystal shows that the 1α-hydroxyl group of $1\alpha,25(OH)_2D_3$ contacts S237 and R274, the 3β-hydroxyl group is coordinated by S278 and Y143, and the 25-hydroxyl group is hydrogen bonded to H397 and H305 (8). These amino acids, except for Y143, are conserved among human, mouse, rat, and chicken VDRs (Fig. 1A). Y143 is replaced with phenylalanine in chicken VDR, which is identical to the corresponding amino acid in PXRs. R274 in hVDR is conserved in VDRs and PXRs, and H397 of hVDR is identical among other VDRs and hPXR. The alignment indicates that S237, S278, and H305 are unique to VDRs and that PXR has other amino acids at these positions. Although the Cα backbones of the VDR and PXR structures are very similar, the presence of a flexible loop between the β-sheet and H7 instead of the H6 found in VDR might be responsible for the ability of PXR to respond to diverse ligands (19) (Fig. 1, B and C). In addition, the crystal structures defined for the VDR-LBD (Δ165–215) and the PXR-LBD (8, 19) indicate that small differences in the amino acid identity of LBD residues cause significant change in the shape of the LBP (Fig. 1). These findings suggest that mutational analysis of VDR based on an amino acid alignment and structural comparison with PXR should be very useful in elucidating the structure-function relationship of VDR and its ligands.

Functional Analysis of VDR Mutants

Site-directed mutagenesis has been a useful tool in the elucidation of structure-activity relationship for NRs and ligands. Replacement of a LBP residue with the corresponding residue of a closely related receptor was used to analyze peroxisome proliferator-activated receptors and estrogen-related receptor-γ (20, 21). Alanine scanning mutagenesis was used to analyze the interaction of VDR with several vitamin D analogs and bile acids (22). To further analyze structure-activity relationships between VDR and two different natural ligands [$1\alpha,25(OH)_2D_3$ and LCA], we replaced ligand-coordinating residues with the corresponding PXR amino acids. We generated hVDR mutants of Y143 and S278, which make hydrogen bonds with the 3-hydroxyl group of $1\alpha,25(OH)_2D_3$, and of S237, which interacts with the 1α-hydroxyl group of $1\alpha,25(OH)_2D_3$. These amino acids were changed to the corresponding amino acids of hPXR (Fig. 1A) or alanine. Vitamin D-resistant rickets-associated mutants of VDR (R274L and H305Q) were also examined (4). Because hPXR F288 contributes to structural differences between VDR and PXR (19), the corresponding amino acid in VDR (S275) was replaced with either phenylalanine or alanine (VDR-S275F and VDR-S275A). The crystal structure of VDR-LBD was determined in a deletion mutant (Δ165–215), because a long flexible loop between helices 1 and 3 prevents the preparation of stable crystals (8). This loop does not seem to contribute to ligand interaction, because VDR (Δ165–215) can be transactivated by $1\alpha,25(OH)_2D_3$, its synthetic analogs, and LCA as efficiently as wild-type VDR (22, 23) (data not shown).

We examined ligand-responsive transcriptional activation by the full-length VDR point mutants. Human

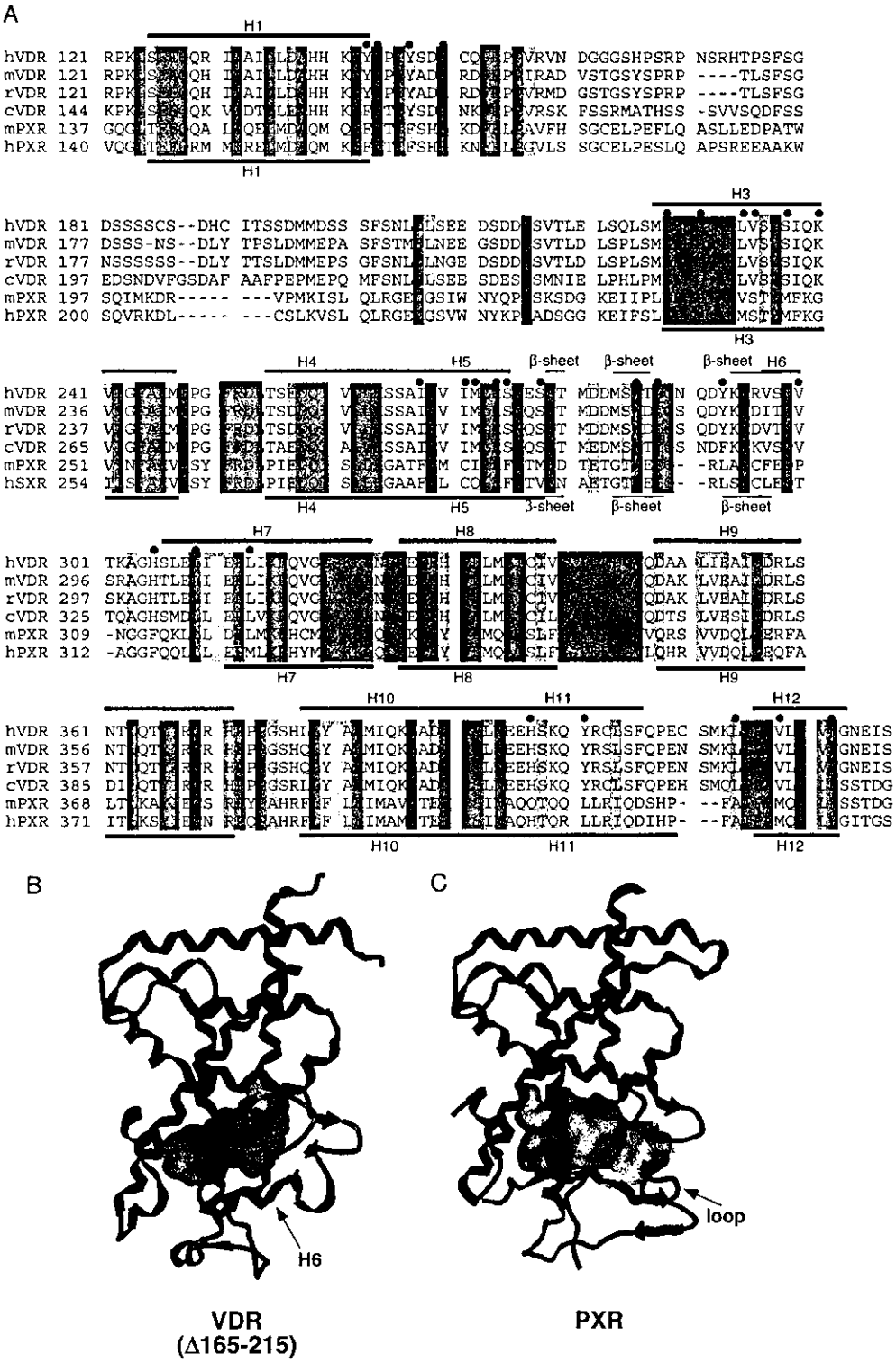

Fig. 1. Comparison of the LBD Sequence of VDR and PXR

A, Sequence alignment of VDR-LBDs [h, human (NCB accession no. AAA61273); m, mouse (NP_033530); r, rat (NP_058754); c, chicken (O42392)] and PXR-LBDs [m, mouse (AAC39964); h, human (AAD05436)]. *Bars* show helices (H) and β-strands in hVDR and hPXR. *Dark shadows* show completely conserved residues in the alignment, and *light shadows* indicate partially conserved residues. *Black circles* show amino acid residues lining the LBP of hVDR. B and C, *Ribbon loop* presentations of hVDR-LBD (Δ165–215) (B) and hPXR-LBD (C). α-Helix, β-sheet, and loop are shown as *ribbons*, *arrows*, and *tubes*, respectively. The LBPs are shown as Connolly channel surfaces.

embryonic kidney (HEK)293 cells were transfected with wild-type VDR or VDR mutants and a luciferase reporter containing a VDR-responsive everted repeat-6 element from the CYP3A4 promoter (10) (Fig. 2A). Because kidney-derived HEK293 cells express endogenous VDR (data not shown), the addition of $1\alpha,25(OH)_2D_3$ or LCA basally induced luciferase activity. Transfection of wild-type VDR effectively increased induction by both ligands. Mutants of residues that coordinate the 3-hydoxyl group of $1\alpha,25(OH)_2D_3$, Y143A, Y143F, S278A, and S278V, were activated by $1\alpha,25(OH)_2D_3$ but not by LCA. $1\alpha,25(OH)_2D_3$ weakly activated S237A and had no effect on S237M activity, whereas LCA activated both mutants. The rickets-causing mutants R274L and H305Q were unresponsive to both $1\alpha,25(OH)_2D_3$ and LCA. S275A maintained responsiveness to $1\alpha,25(OH)_2D_3$ and LCA, but the S275F mutation abolished ligand response. The basal luciferase activity induced by $1\alpha,25(OH)_2D_3$ was repressed by transfection of S237M, R274L, and S275F. This may be due to dominant negative effects of these mutants on endogenous VDR activity through sequestration of retinoid X receptor or cofactors and competitive binding to the vitamin D response element. The data for S237A, S275A, and S278A mutants are consistent with previous data utilizing a reporter with a DR-3 element from the osteopontin promoter (22). In that study, Y143A did not respond to 10 nM

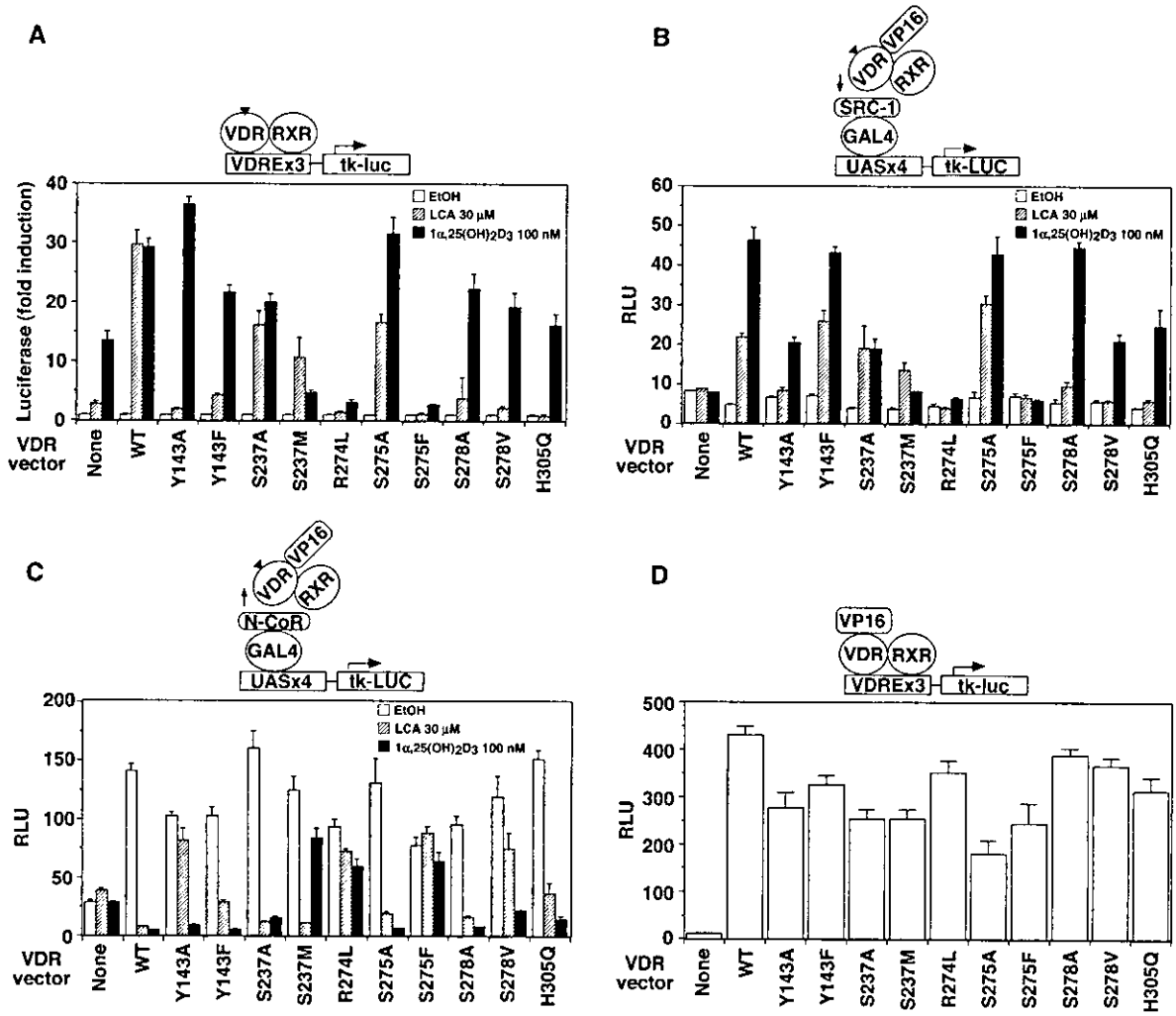

Fig. 2. Functional Analysis of VDR Mutants

A, Activation of VDR and its mutants by LCA and $1\alpha,25(OH)_2D_3$. VDR expression vectors or a control vector and a CYP3A4-ER-6x3-tk-LUC reporter were transfected into HEK293 cells and treated with LCA and $1\alpha,25(OH)_2D_3$. B, Effect of LCA and $1\alpha,25(OH)_2D_3$ on the association between VDR and SRC-1. C, Effect of LCA and $1\alpha,25(OH)_2D_3$ on the association between VDR and N-CoR. Mammalian two-hybrid analysis using GAL4-SRC-1 or GAL4-N-CoR and VP16-VDR was performed in HEK293 cells. D, Evaluation of expression levels of functional VDR mutants in transfected cells. Cells were cotransfected with VP16-VDR mutants or VP16 control vector and CYP3A4-ER-6x3-tk-LUC reporter. The VP16-VDR mutants tested showed similar luciferase values, indicating similar expression of these mutants. Fold induction by the ligands is relative to ethanol (EtOH) vehicle used as a control. RLU, Relative light units. The values represent means ± SD.

$1\alpha,25(OH)_2D_3$. The discrepancy between these results may be due to the difference in ligand concentration or the response element construct tested.

Upon ligand binding, NRs undergo a conformational change that results in the dissociation of corepressors such as nuclear receptor corepressor (N-CoR) and recruitment of coactivators such as steroid receptor coactivator 1 (SRC-1) (2). Ligand-inducible cofactor recruitment was used to further examine ligand response by VDR mutants in the mammalian two-hybrid assay using GAL4-SRC-1 receptor-interacting domain, containing the three LXXLL motifs, and VP16-VDR chimeric expression vectors (10, 12). In the mammalian two-hybrid assay, the NR-interacting fragment of SRC-1 exhibited more robust ligand-dependent interaction with VDR than full-length SRC-1 (data not shown). $1\alpha,25(OH)_2D_3$ strongly induced association of VDR, Y143F, S275A, and S278A, and moderate association of Y143A, S237A, S278V, and H305Q with SRC-1. LCA was able to induce association of VDR, Y143F, S237A, S237M, and S275A with SRC-1 (Fig. 2B). The mammalian two-hybrid assay using the GAL4-N-CoR chimeric corepressor shows $1\alpha,25(OH)_2D_3$-dependent dissociation of N-CoR from VDR, Y143A, Y143F, S237A, S275A, S278A, S278V, and S305Q, as well as LCA-dependent dissociation from VDR, Y143F, S237A, S237M, S275A, S278A, and H305Q (Fig. 2C). Difference in sensitivity of these assays may account for differential responsiveness of VDR mutants to ligands. The data indicate that S237 is more critical for induction by $1\alpha,25(OH)_2D_3$ and that Y143 and S278 are more important for LCA activation. To evaluate the expression of VDR mutants in the cells, we transfected VP16-VDR chimeric mutants together with the VDRE-responsive reporter. Because VP16 chimeric receptors exhibit ligand-independent activity, the luciferase activity gives an indirect measure of the protein expression levels of transfected receptors. All of the VDR mutants show luciferase activities similar to wild-type VDR (Fig. 2D), indicating similar expression of functional VDR mutants in the cells.

The data shown in Fig. 2 suggest that VDR-S278V and VDR-S237M respond selectively to $1\alpha,25(OH)_2D_3$ and LCA, respectively. To further investigate the preference of these VDR mutants for the ligands, dose-response curves were obtained with the GAL4-VDR system, which eliminates the activity of endogenous VDR (Fig. 3). VDR S278A was activated by $1\alpha,25(OH)_2D_3$ as effectively as wild-type VDR. The S278V mutation partially decreased $1\alpha,25(OH)_2D_3$ response. S237M completely abolished $1\alpha,25(OH)_2D_3$ response, whereas S237A had a more moderate effect (Fig. 3A). Importantly, LCA was able to activate S237M as effectively as S237A (Fig. 3B). LCA was unable to activate S278V and weakly activated S278A (Fig. 3B). The data indicate that the S278V mutant is unresponsive to LCA and the S237M mutant loses $1\alpha,25(OH)_2D_3$ response. Therefore, S278V selectively responds to $1\alpha,25(OH)_2D_3$ whereas S237M is specifically activated by LCA.

We examined direct binding of ligands to mutant VDRs *in vitro*. Isotopically labeled $1\alpha,25(OH)_2D_3$ was incubated with glutathione-*S*-transferase (GST)-VDR proteins in the presence or absence of excess unlabeled $1\alpha,25(OH)_2D_3$, and specific binding of $1\alpha,25(OH)_2D_3$ was calculated. $1\alpha,25(OH)_2D_3$ effectively bound to wild-type VDR and S278V but only interacted weakly with S237M (Fig. 4A). $1\alpha,25(OH)_2D_3$ did not bind to GST control protein. The binding of labeled $1\alpha,25(OH)_2D_3$ to wild-type VDR and the S278V mutant was competed by addition of unlabeled $1\alpha,25(OH)_2D_3$ (Fig. 4B). Addition of unlabeled LCA inhibited the interaction of $1\alpha,25(OH)_2D_3$ with wild-type VDR, VDR-S237M, and VDR-S278V, indicating that LCA binds directly to these VDR proteins. For example, 30 μM LCA inhibited binding of 1 nM $1\alpha,25(OH)_2D_3$ to wild-type VDR, VDR-S237M, and VDR-S278V to 74%, 70%, and 79%, respectively, of the control, and 100 μM LCA inhibited those to 59, 38, and 39%, respectively. These findings suggest that the inability of LCA to activate VDR-S278V is not due to loss of binding and is more likely to be caused by a reduction in the ligand-induced shift to an active conformation.

Docking Models of VDR Interacting with LCA

To reveal the molecular basis for the mutated residues in mediating ligand specificity, we generated docking models. As shown by the $1\alpha,25(OH)_2D_3$ model and the VDR crystal structure, S237 hydrogen bonds with the 1α-hydroxyl group, Y143 and S278 interact with the 3-hydroxyl group, and H305 interacts with the 25-hydroxyl group (8) (Fig. 5A). The S237M mutation loses the ability to interact with $1\alpha,25(OH)_2D_3$ (Figs. 2 and 3). The model shows that a methionine residue at position 237 is too close to the 1α-hydroxyl group of $1\alpha,25(OH)_2D_3$ and would be expected to destabilize binding (Fig. 5B). Y143F and S278V do not affect activation by $1\alpha,25(OH)_2D_3$. This may be due to the relatively conservative substitutions in these mutants and an apparent weak contribution of contacts between VDR and the 3-hydroxyl group of $1\alpha,25(OH)_2D_3$ to overall binding energy. S275F mutation causes a marked change in the LBP conformation such that it is unable to accommodate $1\alpha,25(OH)_2D_3$ (Fig. 5B). The mutational analysis in this study confirms the importance of contacts with the 1α-hydroxyl group of $1\alpha,25(OH)_2D_3$ in ligand binding and receptor activation (6, 8).

Because no crystal structure data are available for VDR bound to secondary bile acid ligands, a docking model was generated for LCA in the VDR-LBP (Fig. 5C). The carboxyl group of the LCA side chain is positioned within hydrogen bond distance of H305. Residues Y143 and S278 weakly interact with the 3-hydroxyl group of LCA. Mutation of H305 to glutamine decreased LCA response (Fig. 2), indicating that contacts of Y143 and S278 with LCA are insuffi-

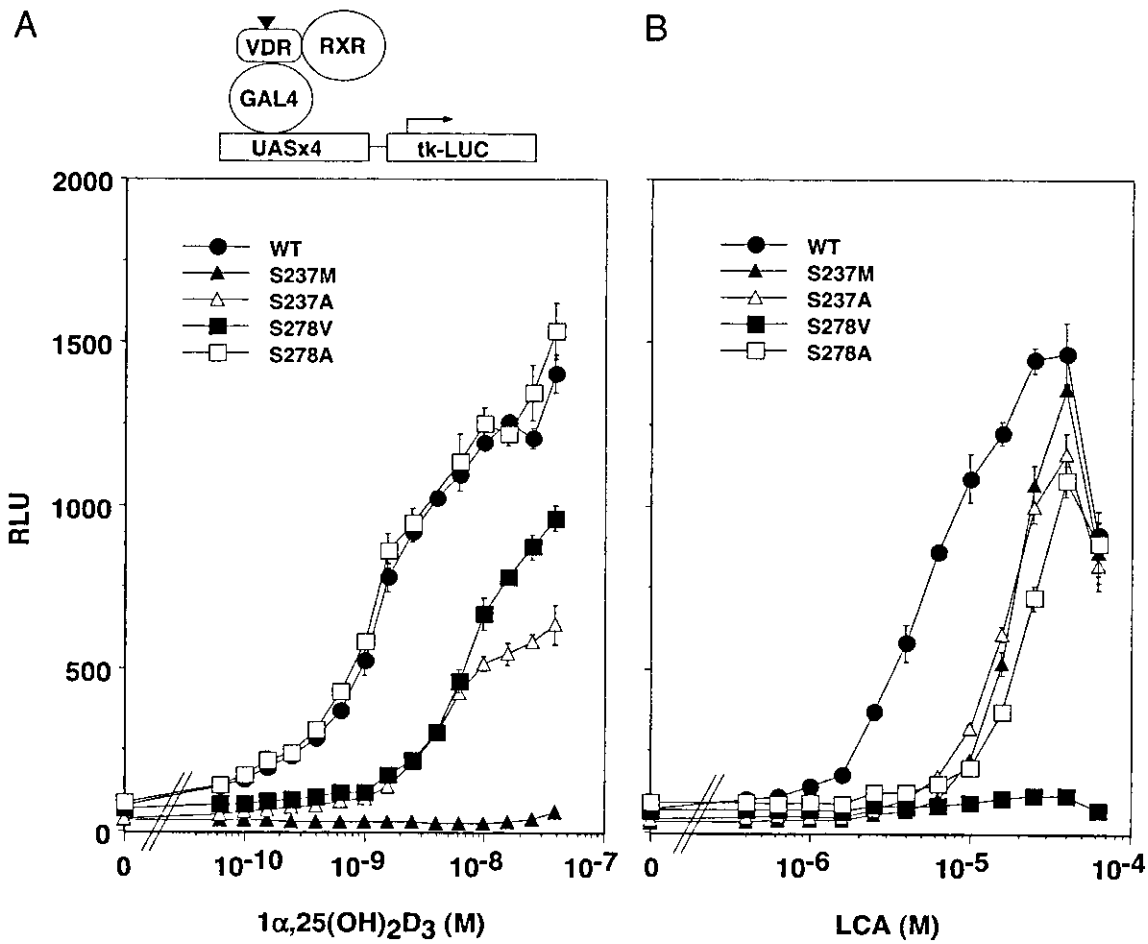

Fig. 3. Dose Response of Wild-Type VDR, S237M, S237A, S278V, and S278A Mutants to $1\alpha,25(OH)_2D_3$ and LCA Using a GAL4-Receptor Luciferase Assay

Cells were cotransfected with GAL4-VDRs and MH100(UAS)x4-tk-LUC reporter. RLU, Relative light units. The values represent means ± SD.

cient for VDR activation. The S278V mutation abolished LCA response, although S278A maintained LCA response (Fig. 3). A valine residue at position 278 would be expected to sterically interfere with the 3-hydroxyl group of LCA, an unfavorable interaction that would be absent in S278A (Fig. 5D). Although the Y143F mutant was responsive, Y143A was not activated by LCA (Fig. 2). The conservative tyrosine to phenylalanine mutation maintains a large aromatic amino acid, whereas the alanine mutation is expected to more severely alter the LBP conformation. S237M mutation abolished $1\alpha,25(OH)_2D_3$ response but did not affect activation by LCA (Fig. 3). As predicted by the docking model, S237 does not interact with LCA, and the S237M mutation has little effect on LCA activity. S275F prevents both LCA and $1\alpha,25(OH)_2D_3$ from docking in the LBP (Fig. 5D). Taken together, these data indicate that LCA and $1\alpha,25(OH)_2D_3$ make nonoverlapping critical interactions with residues of the VDR LBP.

DISCUSSION

VDR (NR1I1) belongs to the NR1I subfamily along with PXR (NRI12) and constitutive androstane receptor [CAR (NR1I3)] (24). PXR is activated by a broad variety of compounds such as xenobiotics, steroid derivatives, and bile acids (17, 18, 25). The PXR crystal structure reveals that polar residues spaced throughout the hydrophobic LBP modulate responsiveness of the receptor to various xenobiotics (19). CAR also functions as a xenobiotic receptor and shares some ligand selectivity with PXR (25). Structural modeling shows that CAR and PXR have a relatively large internal LBP cavity (26). Despite these similarities, CAR has a more restrictive ligand selectivity profile than PXR. The ordered structure of H6 of CAR, which is similar to that of VDR, may impart more narrow ligand selectivity, because PXR has flexible loop 6 in that region (25). Although the cavity of VDR-LBP is smaller than that of PXR or CAR, it is still larger than that of estrogen

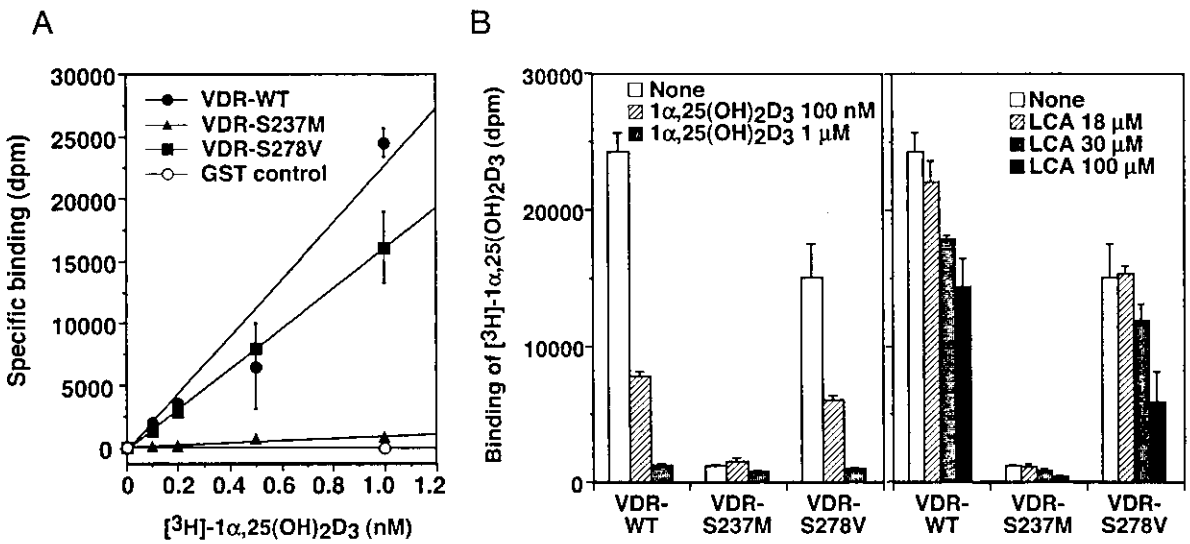

Fig. 4. Ligand Binding Specificity of Wild-Type VDR, VDR-S237M, and S278V Mutants

A, Direct binding of $1\alpha,25(OH)_2D_3$ to VDR. GST-fusion VDR proteins or GST control protein were incubated with increasing concentrations of $[^3H]1\alpha,25(OH)_2D_3$ in the presence or absence of 400-fold excess nonradioactive $1\alpha,25(OH)_2D_3$. B, Competitive binding of $1\alpha,25(OH)_2D_3$ and LCA to wild-type VDR, VDR-S237M, and S278V mutants. GST-fusion VDR proteins were incubated with 1 nM $[^3H]1\alpha,25(OH)_2D_3$ in the presence or absence of the indicated concentrations of nonradioactive $1\alpha,25(OH)_2D_3$ or LCA.

receptor (NR3A1), progesterone receptor (NR3C3), or retinoic acid receptor-γ (NR1B3) (8, 19, 26). CAR and PXR are functionally redundant xenobiotic sensors in that both can regulate common target genes such as CYP3A and CYP2B (27). VDR is also able to regulate CYP3A transcription by binding to the same response element as PXR and CAR (10, 27), suggesting that NR1I receptors have evolved from a common ancestor and have a shared role in mediating the detoxification response to xenobiotics. These findings suggest a potential role of VDR as a xenobiotic sensor and the possibility that VDR responds to natural or synthetic compounds other than vitamin D and bile acid.

VDR is distinct from other NR1I receptors in that it interacts with bile acids with low affinity (at micromolar levels) like PXR and CAR but also responds to an endocrine ligand, $1\alpha,25(OH)_2D_3$, with high affinity, similar to steroid hormone receptors. In this study, we demonstrate that distinct amino acid residues in the LBP are important for interaction with $1\alpha,25(OH)_2D_3$ and LCA. This finding leads to the possibility that $1\alpha,25(OH)_2D_3$ and LCA induce different activated receptor conformations that might recruit distinct sets of cofactors (28). Further analysis of ligand structure-function relationships should be helpful in elucidating the dual functions of VDR as an endocrine receptor for $1\alpha,25(OH)_2D_3$ and as a xenobiotic sensor for secondary bile acids produced by intestinal microflora.

We generated several VDR mutants at residues which hydrogen bond with hydroxyl groups of $1\alpha,25(OH)_2D_3$ and compared their responses to $1\alpha,25(OH)_2D_3$ and LCA in VDRE activation and mammalian two-hybrid assays (Fig. 2). There are some discrepancies in the behavior of VDR mutants in the three assays shown in Fig. 2, A–C. $1\alpha,25(OH)_2D_3$ induced the activation of Y143A as effectively as that of wild-type VDR, but the recruitment of SRC-1 by Y143A was weak. The effect of LCA on Y143F in VDRE activation was very weak, although LCA induced strong interaction with SRC-1 in Y143F as in wild-type VDR. Thus, the VDRE activation by full-length VDR was not completely correlated with recruitment of SRC-1. This may be because NR activation is mediated by sequential interaction with several sets of cofactor complexes (2). LCA induced N-CoR dissociation from Y143F, S278A, and H305Q, but these mutants showed low or undetectable transactivation by LCA in the VDRE-based assay. The data indicate the ligand-inducible dissociation of corepressor is not sufficient to induce VDR transactivation. Transfection experiments using the VDRE activation and mammalian two-hybrid assays in combination are useful in the detection of VDR-ligand interactions and ligand-induced receptor activation. The significance of interaction with particular cofactors on overall VDR transactivation potential requires further investigation.

We found that VDR-S278V and VDR-S237M mutants respond selectively to $1\alpha,25(OH)_2D_3$ and LCA, respectively. These mutants should be useful not only for analysis of structure-activity relationships but also for development of new synthetic VDR agonists. Selective screening using VDR-S278V and VDR-S237M may lead to discovery of a new synthetic ligand that lacks hypercalcemic activity. Recently, mice with humanized PXR or CAR were generated because human PXR and CAR have different ligand selectivity from their rodent homologs (29, 30). Substitution of endogenous VDR with VDR-S278V may produce mice with