Genetic forms of vitamin D–dependent rickets (VDDRs) are due to mutations impairing activation of vitamin D or decreasing vitamin D receptor responsiveness. Here we describe two unrelated patients with early-onset rickets, reduced serum levels of the vitamin D metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, and deficient responsiveness to parent and activated forms of vitamin D. Neither patient had a mutation in any genes known to cause VDDR; however, using whole exome sequencing analysis, we identified a recurrent de novo missense mutation, c.902T>C (p.I301T), in CYP3A4 in both subjects that alters the conformation of substrate recognition site 4 (SRS-4). In vitro, the mutant CYP3A4 oxidized 1,25-dihydroxyvitamin D with 10-fold greater activity than WT CYP3A4 and 2-fold greater activity than CYP24A1, the principal inactivator of vitamin D metabolites. As CYP3A4 mutations have not previously been linked to rickets, these findings provide insight into vitamin D metabolism and demonstrate that accelerated inactivation of vitamin D metabolites represents a mechanism for vitamin D deficiency.
CYP3A4 mutation causes vitamin D–dependent rickets type 3

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Introduction

Vitamin D deficiency rickets is a childhood disorder associated with impaired growth and skeletal mineralization. In most cases, vitamin D deficiency is due to inadequate exposure to sunlight and/or insufficient dietary vitamin D (1). Vitamin D is biologically inactive and undergoes 2 enzymatic conversions to become fully active 1,25-dihydroxyvitamin D. The existence of rickets in areas with abundant sunlight has led to the identification of genetic forms of vitamin D–dependent rickets (VDDRs). Type 1 VDDRs are caused by defects in vitamin D activation, owing to mutations in the genes encoding either the renal 1-α hydroxylase (CYP27B1; VDDR-1A) (2) or the hepatic 25-hydroxylase (CYP2R1; VDDR-1B) (3, 4). Type 2 VDDRs are caused by defects in vitamin D receptor signaling due to mutations in the gene encoding the vitamin D receptor (VDR; VDDR-2A); or heterogeneous nuclear ribonucleoprotein C (HNRNPC; VDDR-2B) (5, 6), a VDR coactivator. Vitamin D homeostasis is also regulated by renal 24-hydroxylase (CYP24A1), an enzyme responsible for 24-oxidation of 1,25-dihydroxyvitamin D3 to inactive calcitriol (7) and degradation of 25-hydroxyvitamin D3 to inactive 24,25-dihydroxyvitamin D3 (8). Here we describe a third kind of VDDR (VDDR-3), in which a gain-of-function mutation in CYP3A4, which encodes a P450 enzyme that metabolizes many xenobiotics and drugs, leads to vitamin D deficiency through accelerated vitamin D metabolite inactivation.

Results and Discussion

Clinical and biochemical studies. We studied 2 unrelated females from non-consanguineous families. Both subjects were born full-term with normal birth weight and length. Proband 1.1 presented to medical attention prior to age 2 years with a history of bowed legs and unsteady gait noted at 20 months. Her pediatrician diagnosed rickets on the basis of bilateral genu varum, poor growth, and reduced serum calcium and phosphorus with elevated serum alkaline phosphatase and parathyroid hormone (Table 1). She was of European ancestry and lived in a sunny climate in Australia. At age 2.5 years she was referred for endocrine evaluation, at which time her height was 81.3 cm (6th percentile) and weight was 11.5 kg (35th percentile). Proband 2.1 was of Middle Eastern descent and did not walk until 4.5 years of age. She was treated for rickets in Jordan, Spain, and the Netherlands prior to evaluation in the United Kingdom at age 16 years, when she had a height of 154.3 cm (9th percentile). Wrist radiographs in both subjects showed features consistent with active rickets (Figure 1A). Both subjects had detectable serum vitamin D3, but low serum levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D (Table 1), which increased only after administration of very large doses of vitamin D or calcitriol and declined rapidly thereafter. After oral administration of 50,000 IU of vitamin D3, proband 1.1 showed a normal increase in serum cholecalciferol, indicating normal absorption of vita-
min D₃, but blunted increases in serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D compared with control subjects with nutritional vitamin D deficiency rickets (Table 1 and ref. 9). Moreover, serum 24,25-dihydroxyvitamin D was low (data not shown), suggesting that increased vitamin D metabolite inactivation by CYP24A1 did not account for apparent vitamin D deficiency. Both subjects required high-dose calcitriol or vitamin D₃ (50,000 IU daily) to maintain normal serum vitamin D metabolites, parathyroid hormone, calcium, and phosphorus. Neither child had a family history of rickets, and all available first-degree relatives had normal circulating vitamin D, metabolites, parathyroid hormone, calcium, and phosphorus (data not shown).

Targeted sequencing identified no mutations in known VDDR1 genes; however, whole exome sequencing revealed a recurrent mutation in CYP3A4. Nucleotide sequences including 5 kb upstream of the transcription start site, as well as exons and exon-intron boundaries for the CYP2R1, CYP27B1, VDR (proband 1.1), and CYP24A1 genes were normal. Whole exome sequencing was performed in probands and parents, and the data were analyzed using models for X-linked, autosomal recessive, and dominant modes of inheritance. No potential candidate gene with 2 mutations was identified; however, each subject had several heterozygous variants potentially causing protein-function alterations. To identify de novo dominant variants, we assumed that disease-causing variants would be rare. Supplemental Table 1 (supplemental material available online with this article; https://doi.org/10.1172/JCI98680DS1) shows surviving candidate variants in the 2 subjects that were absent from their parents. Of these candidates, only CYP3A4 (GenBank NM_017460.5) showed a variant in both subjects, and remarkably, the subjects carried an identical heterozygous single nucleotide change (c.902T>C) that results in replacement of isoleucine by threonine at codon 301 (p.1301T). Sanger sequencing confirmed that both subjects but no available relatives, who were unaffected, carried the missense mutation, indicating the mutation was both recurrent and de novo (Figure 1B). This mutation was not present in public databases or in data from more than 3,000 exomes analyzed at CHOP. Isoleucine 301 is highly conserved (Figure 1C) and forms a critical portion of substrate recognition site 4 (SRS-4), one of 6 SRSs determining CYP3A4 substrate selectivity and product profile (10). SIFT (http://sift.jcvi.org/) and MutPred (http://mutpred.mutdb.org/) predict this change would damage function, but MutPred additionally predicts a possible novel catalytic function. To examine the possibility of a common genetic origin of the mutation, we determined CYP3A4 gene haplotypes in both families. No common haplotype was shared by the 2 mutation carriers, excluding the possibility of a common founder.

### Functional assessment of the CYP3A4 p.1301T mutant.

Because previous work had shown that p.1301 is a determinant of activity for CYP3A4 SRS-4 substrates (11), we hypothesized that threonine replacement of isoleucine 301 might increase oxidation of the vitamin D metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D (12, 13). We tested this hypothesis by analyzing serum 4,25-dihydroxyvitamin D, the principal product of CYP3A4 metabolism of 25-hydroxyvitamin D (14, 15). In both subjects, the ratio of 4β,25-dihydroxyvitamin D to 25-dihydroxyvitamin D was markedly elevated to the range of values exhibited by patients taking rifampin (16), an inducer of CYP3A4 (Table 1). We next used a mammalian cell 2-hybrid expression system in which intracellular 1,25-dihydroxyvitamin stimulates transcription of a luciferase reporter gene (4) to assess the ability of the mutant recombinant CYP3A4 protein to inactivate 1,25-dihydroxyvitamin D₃. Cells expressing mutant compared with WT recombinant CYP3A4 protein had decreased luciferase activity, indicating that p.1301T possessed greater ability to inactivate 1,25-dihydroxyvitamin D₃ (Figure 2A) than WT (P < 0.01 for overall curves; post hoc multiple comparison–adjusted analyses confirmed significance at 0.03 ng/ml [mean difference: 0.13; 95% CI, 0.08–0.22], 0.1 ng/ml [mean difference: 0.72; 95% CI, 0.25–1.20], 0.3 ng/ml [mean difference: 2.03; 95% CI, 1.55–2.51], and 1 ng/ml [mean difference: 3.6; 95% CI, 3.1–4.1]; P < 0.01 for each). Moreover, p.1301T CYP3A4 was more active than CYP24A1, the principal inactivator of 1,25-dihydroxyvitamin D₃, at 0.3 ng/ml (mean difference: 0.42; 95% CI, 0.06–0.77; P < 0.05). We also compared apparent kinetics data between enzymes, as measurements were performed using reporter activity in whole cells. The apparent catalytic efficiency

Table 1. Biochemical analyses

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<thead>
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<th>Normal range</th>
<th>Proband 1.1</th>
<th>Proband 1.1 vitamin D response test</th>
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terol, as this endogenous oxysterol is formed from cholesterol by CYP3A4 and is commonly used as a marker for CYP3A4 activity (17). Both subjects had normal circulating 4β-hydroxycholesterol (Table 1 and ref. 18). We next used a cell-based assay to assess p.I301T CYP3A4 activity for the alternative substrate luciferin IPA. Because the kidney cell line (HEK293T) does not express CYP3A4, we used it to create a cell-based assay. This assay directly measures the conversion of luciferin IPA to luciferin by CYP3A4 and is used to examine CYP3A4 induction (19). Enzymatic activity of both the WT and p.I301T mutant were inhibited by ketoconazole to levels indistinguishable from empty vector (Figure 2D: 1.1 ± 0.1 vs. 1.0 ± 0.1, \( n = 4 \) for each, \( P = 0.39 \)). In contrast to the results for 1,25-dihydroxyvitamin D3 as a substrate, the p.I301T mutant had significantly decreased activity relative to the WT (Figure 2D: 30.0 ± 1.6 vs. 50.1 ± 1.6, \( n = 4 \) for each, \( P < 0.001 \)) for luciferin IPA (19).

We identified a gain-of-function mutation in CYP3A4 in 2 unrelated children with severe rickets due to accelerated vitamin D metabolite inactivation. The observation of identical mutations in the patients, who do not share CYP3A4 haplotypes, makes a founder

\[ \frac{V_{\text{max}}}{K_{\text{m}}} \] of the WT CYP3A4 was 0.014, while that of the p.I301T CYP3A4 mutant was 0.14 and that of CYP24A1 was 0.08. Thus, p.I301T was nearly 10-fold more active than the WT and nearly twice as active as CYP24A1. Because both the WT and mutant CYP3A4 showed increased activity when cotransfected with a cDNA encoding P450 oxoreductase, which localizes to the ER, we believe that the c.I301T substitution does not prevent proper ER localization of CYP3A4.

Increased activity of CYP3A4 p.I301T in transfected cells is not due to increased recombinant protein abundance. To determine whether the increased in vitro activity of p.I301T mutant CYP3A4 might be due to increased protein abundance, we measured recombinant CYP3A4 proteins levels by semiquantitative immunoblotting. The size and abundance of the p.I301T mutant protein were similar to those of the WT (Figure 2, B and C).

CYP3A4 p.I301T does not have increased activity for non–vitamin D substrates. CYP3A4 has 6 substrate domains (10), and the replacement of isoleucine 301 by threonine is predicted to affect only SRS4 substrates. To assay the effect of p.I301T on non–vitamin D substrates, we first examined circulating 4β-hydroxycholesterol, as this endogenous oxysterol is formed from cholesterol by CYP3A4 and is commonly used as a marker for CYP3A4 activity (17). Both subjects had normal circulating 4β-hydroxycholesterol (Table 1 and ref. 18). We next used a cell-based assay to assess p.I301T CYP3A4 activity for the alternative substrate luciferin IPA. Because the kidney cell line (HEK293T) does not express CYP3A4, we used it to create a cell-based assay. This assay directly measures the conversion of luciferin IPA to luciferin by CYP3A4 and is used to examine CYP3A4 induction (19). Enzymatic activity of both the WT and p.I301T mutant were inhibited by ketoconazole to levels indistinguishable from empty vector (Figure 2D: 1.1 ± 0.1 vs. 1.0 ± 0.1, \( n = 4 \) for each, \( P = 0.39 \)). In contrast to the results for 1,25-dihydroxyvitamin D3 as a substrate, the p.I301T mutant had significantly decreased activity relative to the WT (Figure 2D: 30.0 ± 1.6 vs. 50.1 ± 1.6, \( n = 4 \) for each, \( P < 0.001 \)) for luciferin IPA (19).

We identified a gain-of-function mutation in CYP3A4 in 2 unrelated children with severe rickets due to accelerated vitamin D metabolite inactivation. The observation of identical mutations in the patients, who do not share CYP3A4 haplotypes, makes a founder
metabolites 25-hydroxvitamin D and 1,25-dihydroxyvitamin D (15). Although the impact of CYP3A4 on vitamin D homeostasis is limited under physiological conditions, under special circumstances CYP3A4 can have significant effects on vitamin D metabolism. For example, induction of CYP3A4 by anticonvulsants is associated with vitamin D deficiency (15). In addition, targeted induction of CYP3A4 by rifampin provides an alternative clearance mechanism for vitamin D metabolites 25-hydroxvitamin D and 1,25-dihydroxyvitamin D (15).

Figure 2. CYP3A4 (p.I301T) mutant has increased vitamin D degradative activity but decreased activity for other substrates. (A) CYP3A4 (p.I301T) has increased vitamin D degradative activity. CYP3A4 p.I301T had increased inactivation of calcitriol relative to WT (**P < 0.01 by 2-way ANOVA for curve), and post hoc multiple comparison–adjusted analyses confirmed significant differences at 0.03, 0.1, and 0.3 ng/ml (**P < 0.01), and relative to CYP24A1 at 0.3 ng/ml (**P < 0.05) (n = 3 for each). (B and C) There is no difference in the relative abundance of the WT and p.I301T CYP3A4 (n = 3 for each treatment). (B) Immunoblot of cell lysates for CYP3A4 and GAPDH. (C) Quantification of the relative abundance of CYP3A4 and GAPDH. No significant differences were observed between the abundance of the WT and the p.I301T mutant. (D) CYP3A4 (p.I301T) does not have increased catalytic activity for non–vitamin D substrates. The p.I301T mutant had significantly decreased activity for luciferin IPA relative to the WT enzyme (30.0 ± 1.6 vs. 50.1 ± 1.6, n = 4 for each, ***P < 0.001). **P < 0.05. Data are presented as mean ± SEM.

VDDR-1s are caused by mutations in genes encoding proteins that activate vitamin D: CYP2R1 and CYP27B1. VDDR-2s are caused by mutations in genes encoding signal transducing proteins: VDR and HNRNPC. Type 3 is due to gain-of-function mutations in a gene encoding a vitamin D–degrading enzyme: CYP3A4.
that can normalize elevated vitamin D metabolites in patients who lack the native pathway for vitamin D oxidation due to loss-of-function mutations in CYP24A1 (16). Finally, common polymorphisms in CYP3A4 are associated with decreased bone density (20).

Several lines of evidence support our hypothesis that the p.I301T CYP3A4 mutation is the basis for a selective defect in vitamin D metabolism. First, the 2 affected subjects had elevated 4β,25-dihydroxyvitamin D2/25-hydroxyvitamin D ratios but normal 4β-hydroxycholesterol. Second, molecular modeling and in vitro studies previously identified isoleucine 301 as an important determinant of substrate binding; and that replacing isoleucine 301 with another nonconservative residue, phenylalanine, results in 4-fold increases in CYP3A4 activity for some substrates (10, 14). Third, our in vitro studies showed that p.I301T CYP3A4 significantly increased inactivation of 1,25-dihydroxyvitamin D3, but had no effect on clearance of substrate luciferin IPA. Although we did not measure other steroid hormones in the 2 patients, there was no clinical evidence of additional endocrine defects. Therefore, the genetic gain of function in CYP3A4 recapitulated the selective inactivation of vitamin D metabolites well described in patients taking drugs that induce CYP3A4 (21).

These observations lead us to propose that a gain-of-function mutation of CYP3A4 causes a distinct form of VDDR. In contrast to the previously described autosomal recessive forms of VDDR that result from defects in either synthesis of vitamin D metabolites or responsiveness to 1,25-dihydroxyvitamin D, this dominant form of VDDR is due to accelerated inactivation of vitamin D metabolites (schematic of VDDRs in Figure 3). Although nutritional vitamin D deficiency is the most common cause of childhood rickets, these 2 cases, representing the identification of a previously undescribed VDDR, emphasize the importance of considering accelerated vitamin D inactivation as a risk factor for vitamin D deficiency. There is renewed awareness of the importance of daily vitamin D prophylaxis for the prevention of vitamin D deficiency and rickets in infants and children, and our findings highlight the importance of genetic and induced variation in CYP3A4 activity as a modifier of the amount of vitamin D necessary to maintain vitamin D homeostasis.

**Methods**

*Biochemical and molecular analyses*. We measured serum and urine electrolytes, serum creatinine, and parathyroid hormone using routine methods. Measurements of vitamin D$_{25}$, vitamin D$_{25}$, 25-hydroxyvitamin D$_{25}$, 25-hydroxyvitamin D$_{25}$, 24,25-dihydroxyvitamin D, and 4β-hydroxycholesterol were performed by isotope dilution liquid chromatography–tandem mass spectrometry. We measured serum 1,25-dihydroxyvitamin D$_{25}$ and 4β,25-dihydroxyvitamin D$_{25}$ using an ultrahigh resolution chromatographic separation procedure (15) that enables their complete separation.

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