Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D_3

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Steroid hormones are central regulators of a variety of biological processes. According to the free hormone hypothesis, steroids enter target cells by passive diffusion. However, recently we demonstrated that 25(OH) vitamin D_3 complexed to its plasma carrier, the vitamin D-binding protein, enters renal proximal tubules by receptor-mediated endocytosis. Knockout mice lacking the endocytic receptor megalin lose 25(OH) vitamin D_3 in the urine and develop bone disease. Here, we report that cubilin, a membrane-associated protein colocalizing with megalin, facilitates the endocytic process by sequestering steroid–carrier complexes on the cellular surface before megalin-mediated internalization of the cubilin-bound ligand. Dogs with an inherited disorder affecting cubilin biosynthesis exhibit abnormal vitamin D metabolism. Similarly, human patients with mutations causing cubilin dysfunction exhibit urinary excretion of 25(OH) vitamin D_3. This observation identifies spontaneous mutations in an endocytic receptor pathway affecting cellular uptake and metabolism of a steroid hormone.

Steroids destined for intracellular metabolic conversion or binding to nuclear receptors are believed to cross cell membranes by passive diffusion. According to this free hormone hypothesis, steroids bound to plasma carrier proteins are inactive because they cannot reach their intracellular targets (1). However, recent data show that carrier proteins may greatly facilitate steroid uptake by endocytosis of steroid–carrier complexes followed by intracellular release of the steroid (2, 3). Megalin, a member of the low density lipoprotein receptor family abundant in kidney proximal tubules, mediates endocytic uptake of complexes between the steroid 25(OH) vitamin D_3 [25(OH)D_3] and vitamin D-binding protein (DBP) filtered in the glomeruli. The receptor-mediated uptake is required to prevent loss of 25(OH)D_3 in the urine and to deliver the precursor for generation of 1,25(OH)$_2$ vitamin D_3 [1,25(OH)$_2D_3$], a potent regulator of calcium homeostasis and bone turnover. Accordingly, megalin knockout mice lose DBP and 25(OH)D_3 in the urine and develop severe vitamin D deficiency and bone disease (2).

Megalin binds a large number of structurally unrelated ligands, and coreceptors may confer ligand specificity by sequestering and presenting their cargo to megalin (4). For example, intrinsic factor–vitamin B_{12} complex (IF–B_{12}) is taken up in the intestine by a tandem receptor-mediated mechanism; the complex is first bound to a receptor, cubilin, anchored to the outer leaflet of the plasma membrane possibly by an amphipathic helix (5), followed by endocytosis of cubilin and its cargo mediated by megalin (6, 7). The pivotal role of intestinal cubilin is underscored by the vitamin B_{12} deficiency observed in patients with Imerslund–Gräsbeck disease characterized by defective cubilin incapable of binding IF–B_{12} (8). These patients have low molecular weight proteinuria in addition to megaloblastic anemia, indicating dysfunction of cubilin coexpressed with megalin in kidney proximal tubules. However, whereas the role of cubilin in the intestine is well characterized, the physiological role in the kidney remains elusive.

Here, we identify cubilin as an important coreceptor in the endocytic pathway for retrieval of 25(OH)D_3–DBP complexes by megalin-mediated endocytosis in the kidney. We show that absence of cubilin or inhibition of its function markedly reduces cellular uptake of the steroid–carrier complex, and animals or patients lacking functional cubilin are characterized by abnormal vitamin D metabolism. This study identifies patients with mutations in an endocytic pathway that regulates steroid hormone metabolism.

Materials and Methods

Ligands, Receptors, and Antibodies. DBP was purified from human serum (2). Receptor-associated protein (RAP) was produced in *Escherichia coli* (9); ^{3}H-25(OH)D_3 was from Amersham Pharmacia, and 25(OH)D_3 was from Dr. A.-M. Kissmeyer (Leo Pharmaceutical Products, Ballerup, Denmark). Biotin-25(OH)D_3 was synthesized by coupling 25(OH)D_3-3-(3′-aminopropyl)ether (10) with aminocaproic acid-biotin-4-nitrophenyl ester (Pierce) (11). Sterol–DBP complexes were prepared by incubating DBP with 10 to 100-fold excess labeled or unlabeled 25(OH)D_3 (2). Uncomplexed steroid was removed by gel filtration or dialysis. Human retinol-binding protein (RBP) was from Dr. G. Alexander (University of Oslo, Norway). Rabbit megalin and cubilin were purified as reported (5).

The primary antibodies used were rabbit anti-human DBP and anti-human RBP (Dako), goat anti-human DBP (DiaSorin, Stillwater, MN), rabbit anti-rat cubilin (12), and sheep anti-rat megalin (13). Primary antibodies were visualized by using rhodamine-labeled donkey anti-goat IgG (Abcam, Cambridge, U.K.), Cy5-coupled donkey anti-sheep antibody (Amersham Pharmacia), FITC-, tetramethylrhodamine B isothiocyanate-, and horseradish peroxidase-labeled swine anti-rabbit IgGs (Dako), and Alexa$_{488}$ and Alexa$_{546}$ conjugated donkey anti-sheep and goat anti-rabbit antibodies (Molecular Probes). Biotin-25(OH)D_3 was detected by using Cy5-coupled streptavidin (Amersham Pharmacia).

DBP Affinity Chromatography and Western Blotting. A DBP affinity column was generated by immobilizing 5 mg of purified human DBP on cyanogen bromide-activated Sepharose-4B. Chromatography was accomplished by incubating rabbit kidney cortex membranes (6) on the DBP column for 16 h at 4°C, followed by washing with 150 bed volumes of 10 mM Hepes/140 mM NaCl/2 mM $CaCl_2$/1 mM $MgCl_2$/0.6% CHAPS, pH 7.5. Bound proteins were eluted at pH 4.0 with 5 mM EDTA, 1-ml fractions were analyzed

Abbreviations: DBP, vitamin D-binding protein; 25(OH)D_3, 25(OH) vitamin D_3; IF–B_{12}, intrinsic factor–vitamin B_{12} complex; RAP, receptor-associated protein; RBP, retinol-binding protein; SPR, surface plasmon resonance.

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by 4–16% SDS/PAGE, and Western blotting was visualized by using enhanced chemiluminescence.

Surface Plasmon Resonance (SPR) Analysis. Receptor–ligand interactions were assessed by SPR analysis on a BIAcore 2000 (Biosensor) (2). The signal from immobilized proteins corresponded to 51 fmol of immobilized megalin/mm^2 and 42 fmol of immobilized cubilin/mm^2. The signal is expressed in relative response units (RU), i.e., the difference in response between protein and control flow channels. The data were evaluated by using the BIAEVALUATION 3.1 software.

Cell Uptake Studies. BN/MSW cells (14) and human keratinocytes (15) have been described. The cells were grown to confluence in 24-well plates and incubated with ≈50 pM of labeled 25(OH)D_3–DBP in serum-free MEM containing 0.1% ovalbumin. As cell-associated ^{125}I-DBP was negligible, degraded DBP (trichloroacetic acid-soluble radioactivity) was taken as a measure of cellular uptake. Uptake of the steroid was measured as cell-associated radioactivity after washing twice with MEM.

Animal Models. Mixed breed dogs exhibiting autosomal recessive inheritance of cubilin malexpression have been reported (16, 17). Megalin-deficient mice were produced by gene targeting as described (18).

Urine and Blood Samples. Urine from Imerslund–Gräsbeck patients and control individuals was collected and frozen immediately (8). Urine and EDTA plasma were collected from mixed-bred cubilin malexpressing dogs and normal dogs of various strain, age, and sex. For the mice, urine was collected in metabolic cages (19), and EDTA plasma was obtained by retroorbital bleeding. Urine and blood parameters were measured by Nova Medical Medi-Lab (Copenhagen, Denmark) and by Animal Health Diagnostic Laboratory (East Lansing, MI) and analyzed by Student's *t* test unless otherwise indicated. Urinary excretion of 25(OH)D_3 was measured on samples concentrated 20-fold. The displayed values have been corrected accordingly.

Immunostaining of Kidneys and Cells. Dog and mouse kidneys were fixed with paraformaldehyde (19, 20). Semithin cryosections (6) were incubated on glass slides with primary antibody, followed by incubation for 1 h with secondary antibodies.

BN/MSW cells cultured in chamber slides (Nunc) were incubated with 0.5 μM biotin-25(OH)D_3–DBP with or without 5 μM RAP for 30–120 min at 37°C. The cells were washed, incubated with rabbit anti-DBP, followed by tetramethylrhodamine B isothiocyanate-labeled swine anti-rabbit IgG and Cy5-coupled streptavidin. Megalin and cubilin in BN/MSW cells and keratinocytes were visualized by using sheep anti-megalin and rabbit anti-cubilin antisera and Alexa$_{488}$- and Alexa$_{546}$-coupled secondary antibodies. Sections and cells were examined in a Zeiss LSM-510 confocal microscope.

Results

Cubilin Is a Receptor for DBP. Possible coreceptors potentially important for uptake of 25(OH)D_3–DBP complexes in kidney tubules were identified by affinity chromatography using immobilized DBP and solubilized rabbit kidney membranes. Two proteins eluted from the DBP column, but not from a control column (Fig. 1*a*). The ≈600-kDa protein corresponded to megalin in agreement with our previous observation that this receptor mediates uptake of 25(OH)D_3–DBP complexes in the kidney (2). The ≈460-kDa protein was identified as the IF–B_{12} receptor cubilin, thus raising the possibility that cubilin binds DBP. However, as coelution could be due to the previously described interaction between the two receptors (7), we performed experiments using SPR analysis to demonstrate direct binding of DBP to purified cubilin. The results

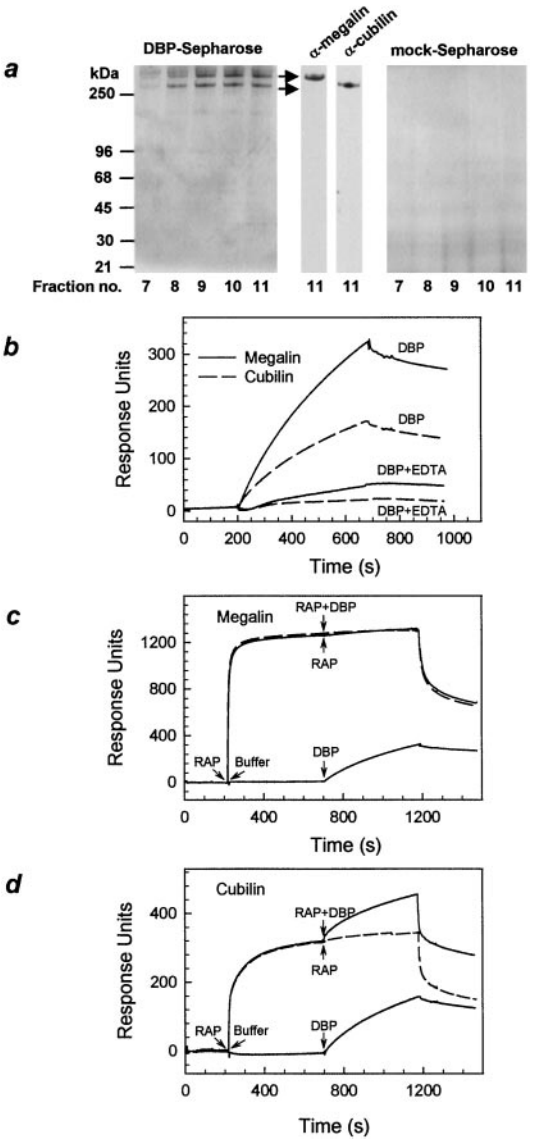

Fig. 1. Characterization of DBP binding to cubilin and megalin. (*a*) DBP affinity chromatography of rabbit kidney membranes. Fractions eluted at pH 4.0 in the presence of 5 mM EDTA from the DBP affinity column (*Left*) or mock column (*Right*) were subjected to SDS/PAGE and silver staining. The two proteins eluted were identified as megalin (≈600 kDa) and cubilin (≈450 kDa) using Western blot analysis of fraction 11 (*Center*). (*b–d*) SPR analysis of DBP (1 μM) binding to immobilized megalin and cubilin. (*b*) The on and off rates were recorded, and the K_d values were 133 nM and 129 nM for binding to megalin and cubilin in the displayed experiment, respectively. EDTA (10 mM) inhibits binding to both receptors. (*c*) RAP (10 μM) was prebound to immobilized megalin at 200–700 s followed by the addition of 0.5 μM DBP (full line) or continuous infusion with 10 μM RAP alone (dashed line). Binding of DBP after preincubation with buffer alone is shown for comparison. (*d*) RAP (10 μM) was prebound to cubilin at 200–700 s, and the experiment was carried out as described for megalin in *c*.

demonstrated Ca^{2+}-dependent binding of DBP to cubilin and confirmed its binding to megalin (Fig. 1*b*). The affinities for DBP binding were similar, with K_d values estimated from five experiments at 110 ± 15 nM for cubilin and 120 ± 27 nM for megalin. The formation of complex with 25(OH)D_3 did not influence binding of DBP to either receptor (not shown).

To discriminate between binding of the steroid–carrier complex to the two receptors, we analyzed the effect of RAP, a 40-kDa endoplasmic reticulum-resident chaperone for megalin, other receptors of the low density lipoprotein receptor family (21), and

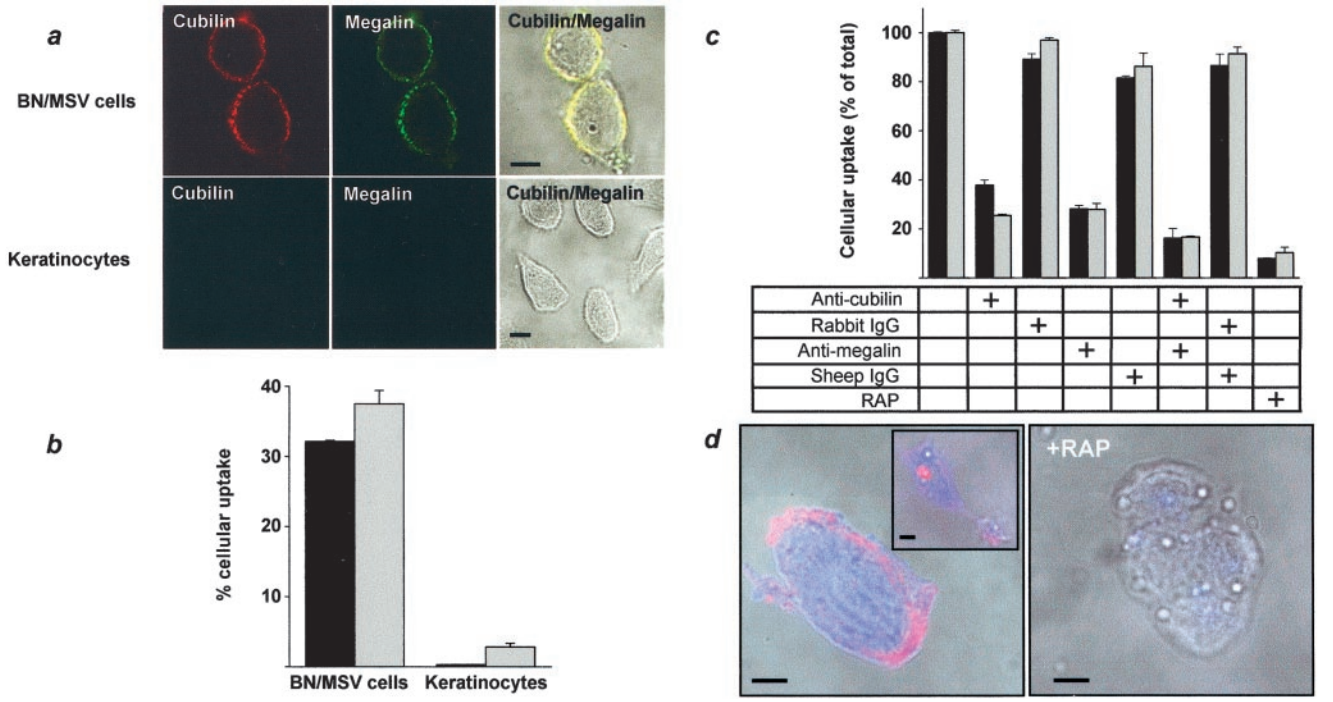

Fig. 2. Cellular uptake of DBP and 25(OH)D_3 in BN/MSV cells and keratinocytes. (*a*) Cellular expression of cubilin and megalin as determined by confocal immunofluorescence microscopy. The panels show cubilin (red) and megalin expression (green) and differential interference contrast images with the fluorescence images superimposed. (*b*) Cells were incubated for 2 h at 37°C with ≈50 pM 25(OH)D_3-^{125}I-DBP or ^{3}H-25(OH)D_3–DBP complex. As cell-associated ^{125}I-DBP was negligible, the percent-degraded DBP was taken as a measure of cellular uptake during the incubation period (black bars). Uptake of steroid was determined as percentage of total ^{3}H-25(OH)D_3 present in the cell lysates (gray bars). Values are means of triplicates ± SD. (*c*) Inhibition of DBP and 25(OH)D_3 uptake in BN/MSV cells. The experiment was carried out as described in *b*, except for the addition of IgG (200 μg/ml) or RAP (2 μM) as indicated. The results are expressed in percent of uptake without additions ± SD. (*d*) Cellular uptake of DBP and 25(OH)D_3 determined by confocal immunofluorescence microscopy. BN/MSV cells were incubated with 200 nM biotin-25(OH)D_3–DBP complex in the absence or presence of 5 μM RAP for 30 or 120 min (*Inset*). DBP staining is depicted in red, biotin-25(OH)D_3 in blue. (Scale bars, 10 μm.)

cubilin (6). RAP binds to multiple sites on megalin and prevents binding of all ligands (4, 21) including cubilin (7), whereas it interacts with a discrete site in cubilin without perturbing binding of the ligand IF–B_{12} (5). Whereas RAP blocked DBP binding to megalin (Fig. 1*c*) in accordance with previous results (2), binding to cubilin remained unperturbed (Fig. 1*d*), indicating binding of DBP and RAP to different sites. Thus, RAP blocks megalin function and uncouples cubilin from trafficking with megalin without interfering with DBP binding to cubilin. This result allowed us to differentiate the contributions of the two receptors to binding and uptake of 25(OH)D_3–DBP complexes in cells.

Cooperation Between Cubilin and Megalin Determines Cellular Accumulation of 25(OH)D_3. We first compared uptake of steroid–carrier complexes in BN/MSV cells that express megalin and cubilin in abundance, with uptake in keratinocytes that lack both receptors (Fig. 2*a*). The complexes were labeled in either the DBP or the 25(OH)D_3 moiety, allowing us to compare cellular uptake of each component (Fig. 2*b*). After 2 h at 37°C, the BN/MSV cells had internalized more than 30% of the DBP and 25(OH)D_3. By contrast, no major uptake was observed in keratinocytes, indicating that passive diffusion of the steroid through the plasma membrane was quantitatively insignificant. To investigate the relative contribution of cubilin and megalin in the uptake process, we measured internalization of 25(OH)D_3–DBP complexes by BN/MSV cells in the presence of antibodies specific to each of the two receptors (Fig. 2*c*). Addition of anti-cubilin inhibited cellular uptake of DBP and 25(OH)D_3 by up to 70%, whereas control IgG had no effect. Anti-megalin antibodies similarly reduced endocytic uptake by ≈70%, thus confirming the importance of megalin for endocytosis of 25(OH)D_3–DBP (2). The combined application of the two IgGs impaired uptake of DBP and 25(OH)D_3 only slightly more (≈80%) than each antibody alone, suggesting that cubilin and megalin function in the same endocytic pathway. As an internal control, uptake of RBP, the plasma carrier for retinol and an established ligand for megalin (22), was not affected by the anti-cubilin antibody, whereas IgG to megalin inhibited endocytosis of RBP by ≈88% (data not shown).

The lack of a transmembrane domain suggests that cubilin needs assistance from megalin to perform endocytosis of its ligand. We therefore applied RAP to inhibit the interaction between cubilin and megalin and to block binding of 25(OH)D_3–DBP directly to megalin (Fig. 2*c*). RAP virtually abolished internalization of the steroid–carrier complex, demonstrating that cubilin activity is not by itself sufficient to target the ligand complex to the interior of the cells. We next used confocal fluorescence microscopy to visualize the internalization process (Fig. 2*d*). Following 30 min of incubation, DBP was present in the endosomal compartment, whereas 25(OH)D_3 appeared in the cytosol. After 2 h at 37°C, a strong perinuclear and lysosomal-like staining for DBP paralleled a considerable cytoplasmic accumulation of the steroid. However, in the presence of RAP, the cellular accumulation of DBP and 25(OH)D_3 was completely inhibited. Thus, the data obtained in BN/MSV cells support a model in which cubilin is the principal binding site on the plasma membrane, followed by association between cubilin and megalin to achieve endocytic uptake of the cubilin-bound 25(OH)D_3–DBP complex.

Cubilin Deficiency in Dogs Causes Disturbances in Vitamin D Metabolism. An unknown autosomal recessive defect causing selective malabsorption of IF–B_{12} and proteinuria, due to failure of apical membrane expression of cubilin in intestine and proximal tubules,

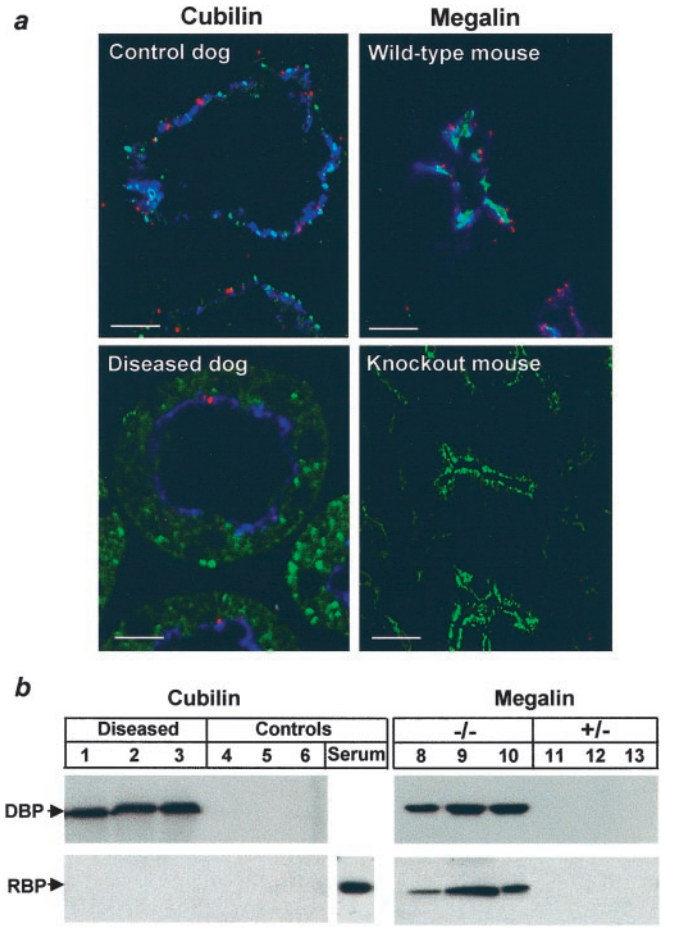

Fig. 3. Reduced endocytic uptake of DBP in cubilin-malexpressing dogs and megalin knockout mice. (*a*) Confocal immunofluorescence microscopy of kidney proximal tubules showing reduced endocytic uptake of DBP in cubilin-diseased dogs. Renal cortical cryosections were stained with sheep anti-megalin, rabbit anti-cubilin, and goat anti-DBP. Megalin is shown in blue, cubilin in green, and DBP in red. Similar stainings were conducted on wild-type and megalin knockout kidneys. (*b*) Urinary excretion of DBP in cubilin-affected dogs. Urine samples from cubilin-affected and control dogs as well as megalin knockouts and control mice were applied to SDS/PAGE followed by anti-DBP and anti-RBP Western blotting. One microliter of dog serum served as positive control for reactivity with canine RBP.

has been demonstrated in a family of giant schnauzer dogs (16). Similar to patients with Imerslund–Gräsbeck disease, these dogs exhibit severe vitamin B_{12} deficiency, unless treated with parenteral vitamin B_{12}. We investigated the role of cubilin for endocytic retrieval of filtered 25(OH)D_3–DBP complexes in dog kidney. All samples from affected dogs were taken during complete hematopoietic remission caused by regular vitamin B_{12} administration. Immunohistology of renal cortical cryosections (Fig. 3*a*) demonstrated that in the affected dogs, in contrast to control dogs, cubilin (green) did not colocalize with megalin (blue) on the apical surface of the proximal tubular epithelium, but was dispersed in vesicles throughout the cytosol and therefore not accessible to ligand in the tubular lumen. In control dogs, DBP antiserum showed a distinct labeling (red) of the endosomal compartment underneath the apical surface of the proximal tubules, indicating uptake of DBP from the glomerular filtrate. In affected dogs, DBP was also present in endocytic vesicles but at a reduced level, indicating that absence of cubilin on the cell surface impaired, but did not entirely abrogate, endocytosis of DBP. For comparison, tubules from megalin knockout mice showed no staining for DBP despite intact expression of cubilin, thus validating the vital role of megalin for the internalization process.

To confirm the reduced uptake of DBP in affected dogs, we collected urine samples and measured excretion of DBP and 25(OH)D_3 (Fig. 3*b* and Table 1). Affected dog urine contained significant amounts of DBP, whereas urine from control dogs did not. Urinary excretion was not due to a secondary effect on megalin activity because RBP, which binds to megalin only, did not accumulate in the urine. For comparison, urine from megalin-deficient mice contained both DBP and RBP. Notably, urinary loss of DBP in five cubilin-deficient animals was accompanied by significant urinary 25(OH)D_3 excretion with concentrations of 0.54 ± 0.19 nM as compared with nondetectable levels in seven control samples, demonstrating that cubilin partakes in the retrieval of filtered steroid (Table 1). In megalin knockouts and wild-type mice, the corresponding values were 0.87 ± 0.01 nM and nondetectable, respectively. As expected, the megalin-deficient mice exhibited significant urinary excretion of retinol (0.25 ± 0.09 μM, $P < 0.01$, $n = 4$), whereas no excretion was detectable in the dogs. Finally, absence of high molecular weight proteinuria (not shown) and normal urinary creatinine concentrations in the receptor-deficient dogs and mice indicated that aberrant glomerular filtration did not account for the urinary excretion of 25(OH)D_3.

To evaluate the physiological significance of reduced 25(OH)D_3 reabsorption for vitamin D homeostasis, blood samples from the same animals were analyzed for vitamin D metabolites (Table 1). Urinary loss of 25(OH)D_3 in the cubilin-affected dogs was accompanied by a 45% reduction in plasma 25(OH)D_3 when compared with control animals (50.6 ± 16.6 nM versus 90.4 ± 30.6 nM, $P < 0.05$). More importantly, the bioactive metabolite 1,25(OH)$_2D_3$ was similarly decreased from 238.1 ± 91.8 pM in wild type to 137.0 ± 32.4 pM ($P < 0.05$) in affected dogs, underscoring the importance of cubilin in vitamin D homeostasis. For comparison, megalin deficiency, which completely disrupts reabsorption of the filtered steroid, caused a ≈70% reduction in plasma levels of both the mono- and dihydroxylated vitamin D_3 metabolites. To confirm the effect of cubilin malexpression on vitamin D_3 metabolism, we subsequently analyzed blood samples from a larger number of dogs. When eight cubilin-deficient animals were compared with 11 controls, the difference in 1,25(OH)$_2D_3$ levels was even more profound, being 211.1 ± 68.1 pM in the affected dogs versus 503.5 ± 154.7 pM in the controls ($P = 0.001$), corresponding to a ≈59% reduction in plasma 1,25(OH)$_2D_3$. Hence, lack of cubilin

Table 1. Urinary excretion of 25(OH)D_3 and plasma vitamin D_3 metabolites in cubilin-diseased and megalin-deficient animals

			Cubilin			Megalin		
	Parameter	Unit	Affected dogs (n = 5)	Control dogs (n = 7)	*P* value	−/− mice (n = 4)	+/− mice (n = 5)	*P* value
Urine	25(OH)D_3	nmol/liter	0.54 ± 0.19	nd	$P < 0.01$*	0.87 ± 0.01	nd	$P < 0.01$*
	Creatinine	mmol/liter	16.33 ± 7.73	16.67 ± 9.02	$P = 0.95$	1.80 ± 1.05	1.77 ± 1.02	$P = 0.96$
Plasma	25(OH)D_3	nmol/liter	50.60 ± 16.61	90.40 ± 30.60	$P < 0.05$	27.50 ± 8.32	86.61 ± 20.40	$P < 0.01$
	1,25(OH)$_2D_3$	pmol/liter	137.01 ± 32.41	238.14 ± 91.80	$P < 0.05$	43.31 ± 17.51	130.04 ± 42.31	$P < 0.01$

nd indicates values below limit of detection; <0.15 nM for 25(OH)D_3.
*Statistical analysis by the Mann–Whitney test.

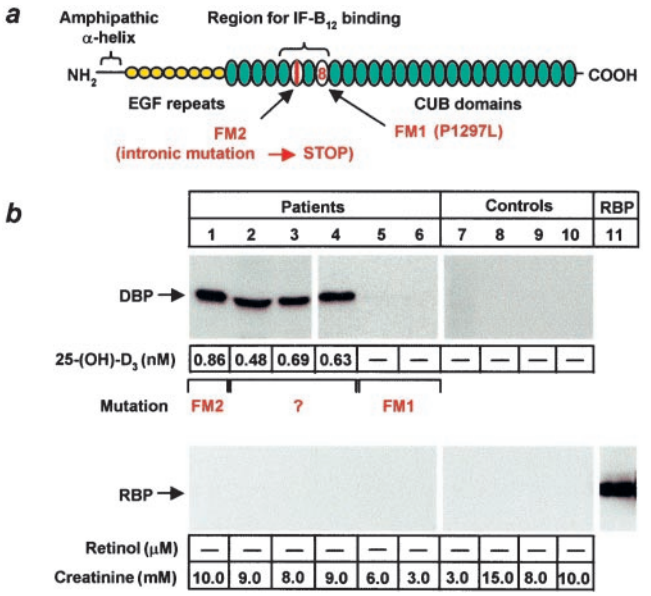

Fig. 4. Urinary excretion of DBP and 25(OH)D_3 in patients with Imerslund–Gräsbeck disease. (*a*) Schematic representation of the structural organization of cubilin. The region for IF–B_{12} binding is depicted together with the FM1 and FM2 mutations in CUB domains 8 and 6. (*b*) Urine samples from six patients and four healthy controls were applied to anti-DBP and anti-RBP Western blot analysis. Purified human RBP (10 ng) was used as positive control. Urinary excretion of 25(OH)D_3 and retinol are shown. Limits of detection were 0.15 nM and 0.1 μM, respectively. The type of mutation present in the cubilin gene is shown. Creatinine is indicated for each patient.

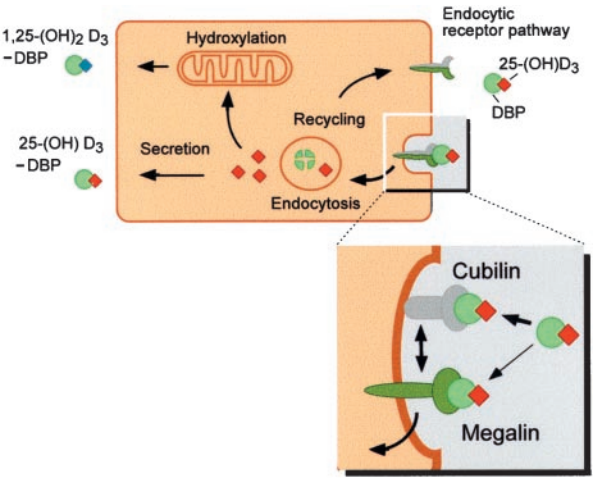

Fig. 5. Schematic model of the tandem function of megalin and cubilin in renal uptake and activation of 25(OH)D_3. Filtered 25(OH)D_3–DBP is endocytosed by the proximal tubular epithelium via the endocytic receptor pathway recognizing DBP. The complexes are delivered to lysosomes where DBP is degraded and 25(OH)D_3 is released to the cytosol; 25(OH)D_3 is either secreted or hydroxylated in the mitochondria to 1,25(OH)$_2D_3$ before release into the interstitial fluid and complex formation with DBP. (*Inset*) Molecular dissection of the endocytic pathway. Cubilin greatly facilitates the endocytic process by sequestering the steroid–carrier complex on the cell surface before association with megalin and internalization of the cubilin-bound 25(OH)D_3–DBP. Some 25(OH)D_3–DBP binds directly to megalin.

activity resulted in vitamin D_3 deficiency in the dogs only slightly milder than that observed in megalin knockout mice, thus confirming the physiological importance of cubilin in this tandem receptor pathway.

Patients with Mutations in the Cubilin Gene Exhibit Urinary Loss of DBP and 25(OH)D_3. Finally, we collected urine from patients with Imerslund–Gräsbeck disease to confirm the validity of the dog model in humans. Cubilin consists of a cluster of 27 CUB domains preceded by eight epidermal growth factor repeats and a putative amphipathic helix. Two mutations in the cubilin gene (FM1 and FM2, respectively) were recently identified in the Finnish population (8) (Fig. 4*a*). Urine samples from six patients exhibiting megaloblastic anemia because of selective intestinal vitamin B_{12} malabsorption and four control subjects were analyzed by SDS/PAGE and Western blotting (Fig. 4*b*). Similar to the animal model, four patients excreted large amounts of DBP and 25(OH)D_3 as compared with controls, whereas two patients did not excrete the metabolite. Interestingly, this difference depended on the nature of the mutation in the cubilin gene (Fig. 4*a*). Urinary loss of DBP and 25(OH)D_3 is seen in patient 1 homozygous for the FM2 mutation, which is an intronic single nucleotide substitution causing the activation of a cryptic splice site, leading to an insertion in CUB domain 6, and an in-frame integration of multiple stop codons (8). As a consequence, patients carrying this mutation are expected to express a truncated form of the receptor. In contrast, patients with the FM1 mutation (patients 5 and 6) have a missense mutation (8) causing a single amino acid substitution in the binding site for IF–B_{12} in CUB domain 8. The ability of the FM1 patients to reabsorb DBP normally suggests that the binding site for 25(OH)D_3–DBP is distinct from the binding site for IF–B_{12}. The mutation status of patients 2, 3, and 4 is not known, but their mutations obviously also affect binding of DBP to the receptor demonstrated by urinary loss of 25(OH)D_3 and DBP. Because no other mutations affecting IF–B_{12} metabolism have been disclosed in the cubilin gene, these patients may exhibit a defect in one or more unknown genes required for proper processing and sorting of cubilin (8), as was demonstrated in the cubilin-deficient dogs (17). None of the six patients exhibited urinary excretion of RBP and retinol, indicating intact megalin function. Furthermore, glomerular filtration was not different between patients and control individuals as determined by absence of high molecular weight proteinuria (data not shown) and comparable urinary excretion of creatinine.

Discussion

Steroid hormones regulate important biological processes including reproduction, metabolism, and skeletal formation. Surprisingly, the mechanism providing delivery of these hydrophobic molecules into target cells remains unclear. Although some uptake may be accounted for by diffusion of the free steroid (1), this process lacks specificity and is restricted by the small fraction of uncomplexed steroid in the circulation. Endocytic receptor pathways may therefore have evolved to target steroid–carrier complexes to cells with large requirements, e.g., for metabolic conversion of the steroids. The present study demonstrates that cubilin and megalin constitute a functional unit for delivery of 25(OH)D_3 to renal proximal tubule cells.

Cubilin was first identified as a receptor for IF–B_{12} complex in the terminal ileum (6). As direct association between cubilin and megalin has been demonstrated (7), it was suggested that molecular cooperation provides the basis for internalization of ligands bound to cubilin. Thus, cubilin with bound ligand may undergo megalin-mediated endocytosis, unload its cargo in lysosomes, and recycle back to the plasma membrane together with megalin (7, 23).

Our data show that although 25(OH)D_3–DBP can bind directly to megalin, cubilin greatly facilitates the endocytic process by sequestering the steroid–carrier complex on the cell surface before internalization via megalin. This pathway rescues steroid from urinary excretion and ensures sufficient substrate for generation of 1,25(OH)$_2D_3$ by the 1α-hydroxylase (Fig. 5).

The importance of cubilin in the endocytic process is underscored by the 70% reduction in $25(OH)D_3$–DBP uptake in BN/MSV cells following inhibition of cubilin function. The physiological importance is demonstrated by the consistent differences in plasma vitamin D_3 parameters between controls and cubilin-deficient dogs, even though animals of both groups were housed and fed differently and were not genetically defined except with regard to the cubilin malexpression locus. However, no statistically significant difference in PTH levels was found between cubilin-affected and control dogs (4.8 ± 1.8 pM versus 3.5 ± 0.9 pM, respectively; $P = 0.14$, $n = 10$), indicating that the decrease in plasma $1,25(OH)_2D_3$ levels did not cause secondary hyperparathyroidism. However, all dogs were on high quality diets, and it is possible that changes in PTH levels due to cubilin malfunction may only be observed when dietary vitamin D intake is suboptimal. It is evident that environmental factors interact with genetic determinants of nutrient utilization to produce normal or abnormal states of health. For instance, mutations in the sodium/iodide symporter expressed in thyroid follicular cells that do not cause hypothyroidism when there is sufficient dietary iodine may become clinically apparent only when dietary iodine intake is suboptimal (24).

In the cubilin-deficient dogs, plasma $1,25(OH)_2D_3$ was reduced $\approx$59% as compared with about 70% in megalin-deficient mice. Although cubilin has previously been identified as a receptor for albumin, apolipoprotein A1, and Clara cell secretory protein in the kidney (19, 20, 25), the overall consequences of cubilin deficiency in metabolism of these ligands remain unclear. To our knowledge, $25(OH)D_3$ complex represents the only ligand for renal cubilin associated with changes in important physiological parameters such as plasma $1,25(OH)_2D_3$ levels. We therefore propose that a crucial function of renal cubilin is to participate in vitamin D retrieval and metabolism.

In view of the predominant expression and ascribed role of the 1α-hydroxylase in the renal proximal tubules, it is surprising that megalin-deficient mice, which cannot mediate endocytic uptake of filtered $25(OH)D_3$–DBP complexes (2), are not devoid of calcitriol. However, this may be explained by extrarenal production of $1,25(OH)_2D_3$. 1α-Hydroxylase has been identified in skin, monocytes, and lymphocytes, but the biological relevance in vitamin D homeostasis has remained elusive (26). The present results indicate that although the kidney is the quantitatively most important site for of 1α-hydroxylase activity, extrarenal tissues seem to produce calcitriol, at least when production in the proximal tubules fails, as in the megalin- and cubilin-deficient animals.

A detailed analysis of the vitamin D homeostasis in Imerslund–Gräsbeck disease, as compared with suitable controls, was precluded by the limited number of patients and the fact that plasma calcitriol levels vary with diet and seasons. However, patients exhibiting urinary loss of DBP and $25(OH)D_3$ are likely to experience reduced plasma $1,25(OH)_2D_3$ levels, as did the cubilin-deficient dogs. Interestingly, nonspecific rheumatic symptoms like diffuse bone pain, muscle ache, and fatigue may develop as the result of even moderate reductions ($\approx$40%) in plasma $25(OH)D_3$ and $1,25(OH)_2D_3$ levels without biochemical or clinical signs of osteopathy (27). Such symptoms may easily be overlooked or considered a result of the anemia in Imerslund–Gräsbeck patients. Future studies of hypovitaminosis D patients may reveal other mutations in the cubilin gene that selectively affect binding and uptake of $25(OH)D_3$–DBP.

In conclusion, cubilin is a physiologically important coreceptor for megalin-mediated delivery of the steroid $25(OH)D_3$ to kidney epithelial cells, and we identify mutations in an endocytic pathway leading to abnormal steroid metabolism. Similar endocytic pathways may exist for other steroid hormones, and identification of such pathways may have important clinical and pharmaceutical implications.

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