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An Endocytic Pathway Essential for Renal Uptake and Activation of the Steroid 25-(OH) Vitamin D_3

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Summary

Steroid hormones may enter cells by diffusion through the plasma membrane. However, we demonstrate here that some steroid hormones are taken up by receptor-mediated endocytosis of steroid-carrier complexes. We show that 25-(OH) vitamin D_3 in complex with its plasma carrier, the vitamin D–binding protein, is filtered through the glomerulus and reabsorbed in the proximal tubules by the endocytic receptor megalin. Endocytosis is required to preserve 25-(OH) vitamin D_3 and to deliver to the cells the precursor for generation of 1,25-$(OH)_2$ vitamin D_3, a regulator of the calcium metabolism. Megalin$^{-/-}$ mice are unable to retrieve the steroid from the glomerular filtrate and develop vitamin D deficiency and bone disease.

Introduction

Vitamin D_3 is a steroid hormone that plays an important role in regulation of the systemic calcium and bone metabolism. It is produced in the skin from the precursor 7-dehydrocholesterol and released into the circulation. Subsequently, vitamin D_3 undergoes two sequential hydroxylation reactions: first in the liver to 25-(OH) vitamin D_3 and then in the kidney to 1,25-$(OH)_2$ vitamin D_3. The latter is the active form of the vitamin that acts through binding to nuclear vitamin D receptors expressed in various target tissues. In particular, 1,25-$(OH)_2$ vitamin D_3 induces intestinal absorption of calcium from the diet to increase systemic Ca^{2+} concentrations.

Like other steroid hormones, vitamin D_3 metabolites are lipophilic compounds transported in the circulation by plasma carrier proteins. The 58 kDa vitamin D–binding protein (DBP) is the principal transporter for vitamin D molecules and exhibits highest affinity for 25-(OH) vitamin D_3 (K_d of 10^{-10}–10^{-12} M) (Haddad et al., 1981; Haddad, 1995). Due to this tight binding and the high plasma concentration of DBP (0.3–0.5 mg/ml), virtually all 25-(OH) vitamin D_3 molecules in the circulation are present in a complex with DBP. Only approximately 0.003% of the metabolite is found in free form (Bikle et al., 1986).

The regulated step in generation of active vitamin D is the conversion of 25-(OH) vitamin D_3 to 1,25-$(OH)_2$ vitamin D_3 in the kidney. The cell type responsible for this activation is the epithelial cell of the proximal tubule. This cell type takes up the precursor 25-(OH) vitamin D_3 and converts it into the active vitamin by action of the 25-(OH) vitamin D_3 1α-hydroxylase in the mitochondria (Takeyama et al., 1997). Considerable interest has focused on elucidating the specific mechanisms that deliver 25-(OH) vitamin D_3 to this specialized cell type. In particular, the mode of uptake of the steroid and the role of DBP in this process is still unclear. According to the "free hormone hypothesis," it is the free 25-(OH) vitamin D_3 that is taken up into the tubular epithelium. Uptake is believed to proceed from the basolateral site of the tubule by yet unknown mechanisms (Mendel, 1989; Haddad, 1995).

Recently, a major pathway for the uptake of ligands from the luminal site into the proximal tubules was identified. Megalin is a multifunctional clearance receptor expressed in the neuroepithelium and in proximal tubular cells of the kidney. The protein shares extensive structural similarities with the low density lipoprotein (LDL) receptor and is a member of the LDL receptor superfamily (Saito et al., 1994). Like other members of this gene family, megalin exhibits broad ligand specificity and mediates the uptake and lysosomal degradation of numerous macromolecules. Ligands for megalin identified in vitro include lipoproteins, proteases, and protease inhibitors (Willnow et al., 1992; Moestrup et al., 1995, 1998; Stefansson et al., 1995). The expression of megalin on the luminal surface of the tubular epithelium and its ability to take up ligands injected into the proximal tubules suggests a role for the receptor in the clearance of filtered macromolecules (Moestrup et al., 1995, 1996). The nature of endogenous ligands taken up by megalin in vivo remains unclear.

To elucidate the physiological significance of megalin, we recently generated mice deficient in the encoding gene. Most megalin-deficient mice die perinatally from holoprosencephaly, a developmental defect of the forebrain (Willnow et al., 1996). The exact causes underlying this defect have not been elucidated, but the severity of the phenotype varies among individual animals, and 1 in 50 of the megalin$^{-/-}$ mice survive to adulthood. Surviving knockout animals were used to study the role of the receptor in the renal proximal tubules. Unexpectedly, we found that complexes of 25-(OH) vitamin D_3 and DBP are filtered through the glomerulus and reabsorbed by megalin into the proximal tubular cells. Abnormal urinary excretion of 25-(OH) vitamin D_3 and DBP in megalin knockout mice results in severe vitamin D deficiency and bone disease. Thus, we have identified

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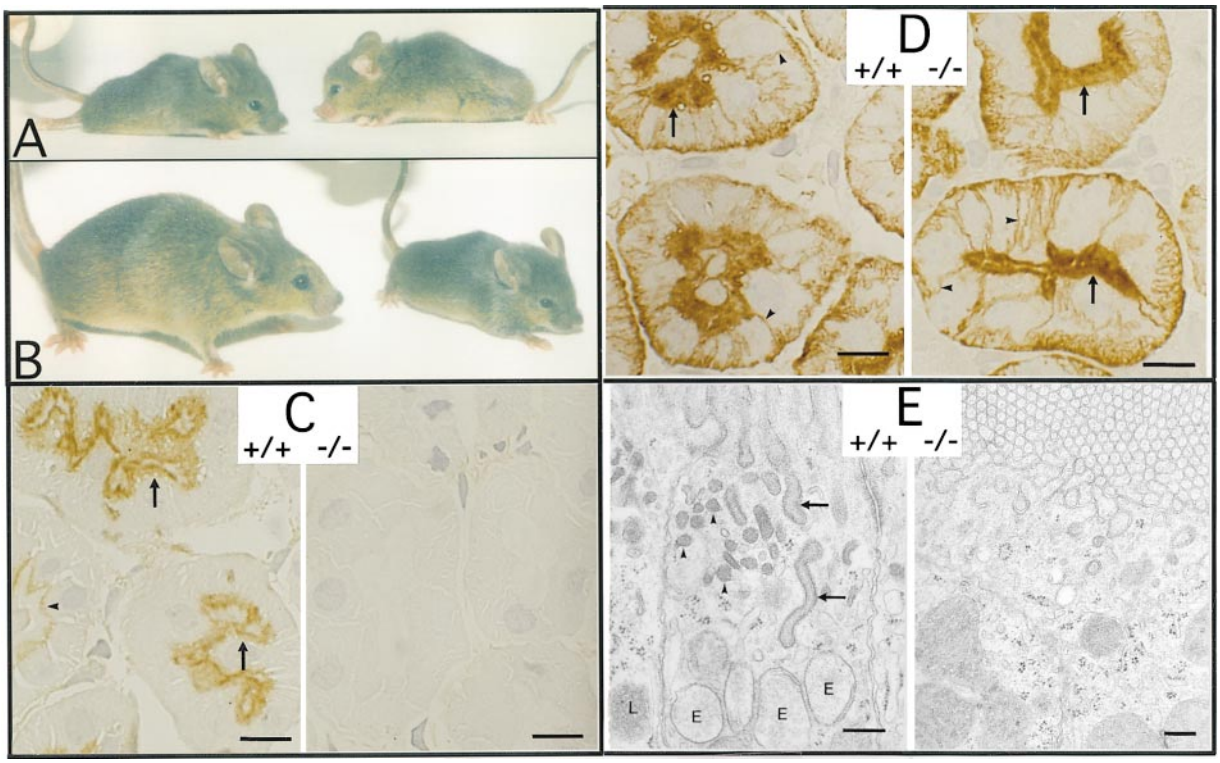

Figure 1. Appearance and Kidney Histology of Wild-Type and Megalin$^{-/-}$ Mice

(A) Two megalin$^{-/-}$ mice are shown at 6 weeks of age. Notice facial malformations indicating holoprosencephaly.

(B) Depicted are a megalin$^{-/-}$ mouse (right) and wild-type littermate.

(C and D) The immunohistochemical detection of megalin (C) and aquaporins (D) in wild-type (+/+) and megalin$^{-/-}$ kidneys (−/−) is shown. Semithin cryosections of tissues fixed in 4% paraformaldehyde were incubated with primary antibodies followed by peroxidase-conjugated secondary antibody. Expression of megalin and aquaporins on the apical surface of the epithelium is indicated by arrows; arrowheads denote basolateral expression of aquaporins. Bars = 10 μm.

(E) Electron micrographs of sections through proximal tubules of wild-type and megalin-deficient kidneys are shown. Tissues were fixed in 4% paraformaldehyde and 1% glutaraldehyde, postfixed in OsO_4, dehydrated, and embedded in Epon prior to routine sectioning. Arrowheads indicate dense apical tubules; arrows indicate coated pits. E, endosomes; L, lysosomes. Bars = 0.25 μm.

a renal uptake pathway that is essential to preserve vitamin D metabolites and to deliver the precursor for generation of 1,25-$(OH)_2$ vitamin D_3.

Results

Urinary Excretion of Vitamin D–Binding Protein in Megalin$^{-/-}$ Mice

Approximately 2% of megalin knockout animals survive to adulthood. These animals are severely retarded in growth (Figures 1A and 1B). Superficially, kidneys from the adult megalin$^{-/-}$ mice were indistinguishable from those of wild-type controls. In particular, the lack of receptor expression in the proximal tubular epithelium (Figure 1C) did not affect expression of other cell surface proteins such as aquaporins (Figure 1D), insulin-like growth factor-II receptor, or ecto-5′-nucleotidase (not shown). However, electron microscopical analysis demonstrated that components of the endocytic apparatus, including endosomes, lysosomes, and recycling vesicles (dense apical tubules) were significantly reduced in number (Willnow et al., 1996). This result suggested a deficiency in the uptake of filtered macromolecules in proximal tubules lacking megalin (Figure 1E). To test this hypothesis, we analyzed the urine of megalin-deficient mice by SDS polyacrylamide gel electrophoresis (PAGE) and compared it to control samples (Figure 2). No proteins larger than serum albumin (68 kDa) were present in the urine, demonstrating normal glomerular function in megalin$^{-/-}$ animals. However, knockout mice specifically excreted several low-molecular-weight proteins that were not found in control samples (Figure 2A). Such low-molecular-weight proteinuria indicates an inability of the proximal tubules to reabsorb small plasma proteins filtered through the glomerulus. To identify these proteins, which are potential ligands for megalin, an amino-terminal peptide sequence was derived from the major 58 kDa protein found in knockout urine (Figure 2A). The sequence obtained was identical to the first eight amino acids (L E R G R D Y E) of the mature mouse DBP (Yang et al., 1990). DBP was present exclusively in urine from megalin-deficient but not control animals (Figure 2B).

Megalin Is the Renal DBP Receptor

To test directly whether megalin binds DBP, the carrier was purified from human serum and from urine of patients with Fanconi syndrome, who are known to excrete

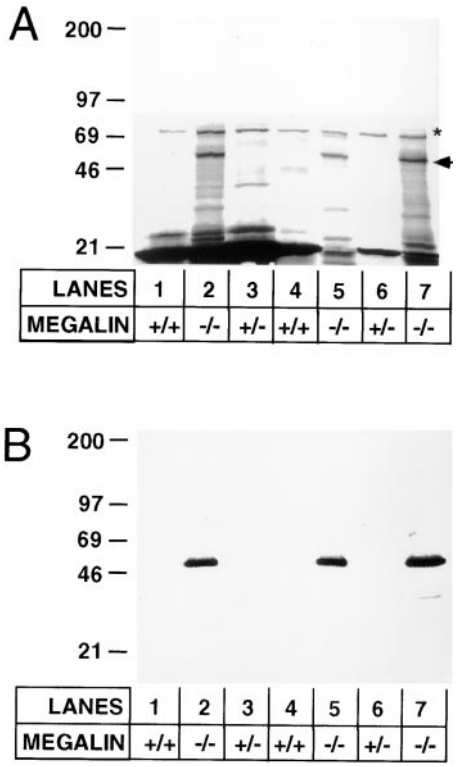

Figure 2. Urinary Protein Profile of Wild-Type and Megalin$^{-/-}$ Mice

(A) Fifteen microliters of urine from mice of the indicated genotypes were subjected to 4%–15% nonreducing SDS-PAGE and staining with Coomassie. Protein bands corresponding to serum albumin (asterisk) and DBP (arrowhead) are highlighted.

(B) Urine samples as in (A) were subjected to immunoblot analysis using anti-DBP antiserum and the enhanced chemiluminescence system (ECL).

DBP (Figure 3A, inset). In BIAcore experiments, urine and serum DBP bound specifically to megalin with a K_d of 108 nM $\pm$ 62 nM (standard error of the mean, n = 4) (Figure 3A). Binding was dependent on calcium, a characteristic feature of ligand binding to LDL receptor–related receptors. Complexes of DBP and 25-(OH) vitamin D_3 formed in vitro bound to the receptor with similar affinity as DBP alone (K_d of 111 μm $\pm$ 44 μm, n = 4, data not shown). To show that megalin is the only cellular binding site for DBP in the kidney, cryosections of wild-type mouse kidneys were incubated with purified DBP. Binding of the protein colocalized with megalin on the apical surface of the proximal tubular cells. No binding was seen in megalin-deficient kidneys (Figure 3B). When anti-DBP antiserum was added to wild-type kidney sections, endogenous DBP was detected in endosomes and lysosomes, indicating uptake from the glomerular filtrate. In contrast, no uptake of endogenous DBP was detected in knockout kidneys (Figure 3C). The complete absence of DBP binding sites and lack of uptake of endogenous DBP in knockout kidneys indicated that megalin is the only receptor to retrieve the protein from the glomerular filtrate.

Megalin Mediates Tubular Uptake of DBP and 25-(OH) Vitamin D_3/DBP Complexes

To show that megalin mediates endocytosis of DBP from the glomerular filtrate, ^{125}I-DBP was infused into

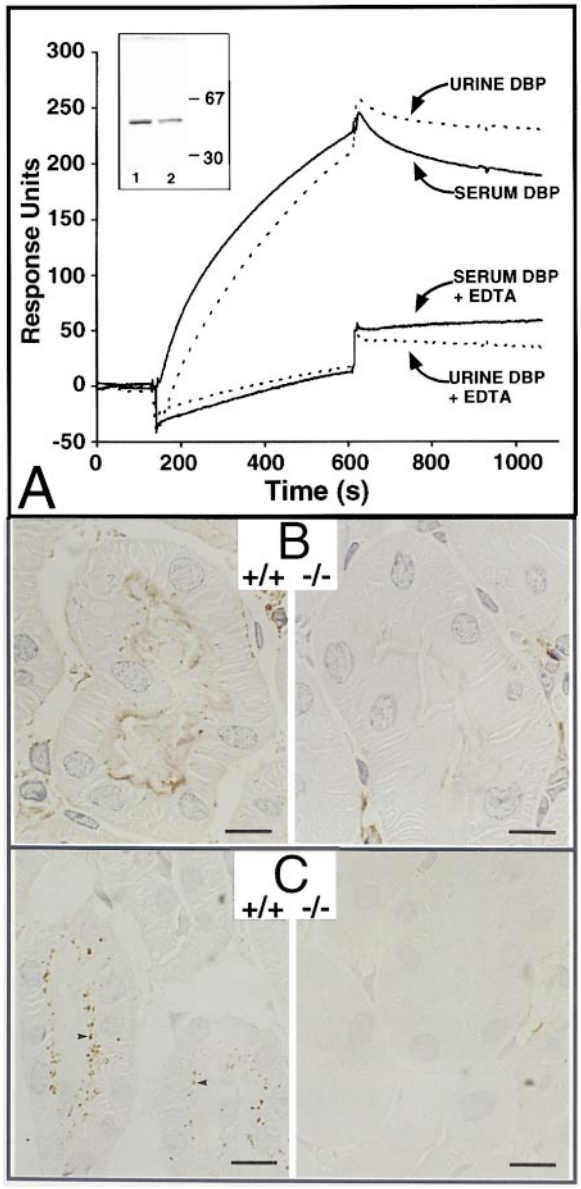

Figure 3. Analysis of DBP Binding to Megalin

(A) DBP purified from human serum or urine of patients with Fanconi syndrome was incubated with immobilized megalin in the presence or absence of 20 mM EDTA. Binding to the receptor was detected by surface plasmon resonance signal (BIAcore) as described in Experimental Procedures and is indicated in response units. (Inset) Preparations of DBP from human serum (lane 1) and urine (lane 2) are depicted. DBP was purified by immunoaffinity chromatography using anti-DBP antiserum coupled to CNBr-activated sepharose. One microgram of DBP was subjected to 10% reducing SDS-PAGE and staining with $AgNO_3$.

(B) Cryosections through wild-type and megalin$^{-/-}$ kidneys were incubated with purified serum DBP, and cell surface binding of DBP was detected using rabbit anti-DBP antiserum and peroxidase-conjugated anti-rabbit IgG. Bars = 10 μm.

(C) Cryosections through wild-type and megalin$^{-/-}$ kidneys were incubated with anti-DBP IgG, followed by peroxidase-conjugated anti-rabbit IgG. Arrowheads indicate endogenous DBP present in endosomes and lysosomes of wild-type kidneys. Bars = 10 μm.

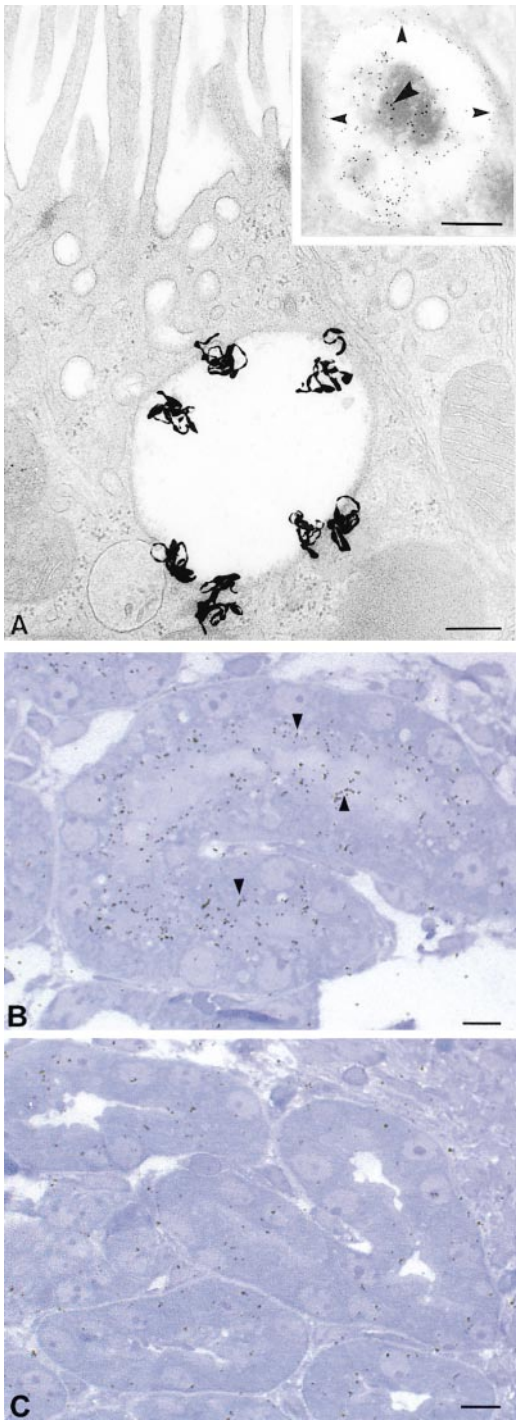

Figure 4. Inhibition of Proximal Tubular Uptake of ^{125}I-DBP by the Receptor-Associated Protein

(A) Individual rat proximal tubules were microinfused in vivo with 50 nl of phosphate buffer containing 0.5–2.7 pmol ^{125}I-DBP (1000 cpm total) in the absence or presence of 0.4 mg/ml RAP. The amount of tubular uptake of ^{125}I-DBP was determined as depicted in Table 1. Then the kidneys were prepared for routine sectioning as in Figure 1E and the electron micrographs subjected to autoradiography. In the absence of RAP, ^{125}I-DBP was detected in apical endosomes of proximal tubular cells (shown). No cellular uptake of ^{125}I-DBP was observed in the presence of RAP (not shown). Bar = 0.25 μm. (Inset) The inset illustrates lysosomal accumulation of DBP by incubation of ultrathin sections with rabbit anti-DBP and mouse anti-LAMP1 antibodies followed by gold anti-rabbit and anti-mouse IgG. DBP, large gold particles, large arrowheads; LAMP1, small gold particles, small arrowheads.

(B and C) Semithin cryosections through wild-type (B) and megalin knockout kidneys (C) were incubated with ^{125}I-labeled RAP and subjected to autoradiography. The arrowheads denote ^{125}I-RAP bound to the luminal surface of proximal tubular cells in wild-type kidneys (B). No RAP binding was seen in megalin-deficient kidneys (C). Bars = 5 μm.

Table 1. Uptake of ^{125}I-DBP into Perfused Rat Proximal Tubules

Ligand	Percent Renal Uptake (±SEM)	n
^{125}I-DBP	75.0 ± 11.3	8
^{125}I-DBP + RAP	8.2 ± 10.4	8

Percent of total radioactivity taken up into proximal tubules is indicated. For details, see Experimental Procedures. n, number of injected tubules; SEM, standard error of the mean.

proximal convoluted tubules of anesthetized rats and the amount of tubular uptake of the tracer was determined. In these experiments, a total of 75% of the injected ligand was taken up (Table 1). The internalized DBP was detected in intracellular vesicles that also contained LAMP1, a lysosomal marker protein, suggesting that the protein was delivered to lysosomal degradation (Figure 4A). To show that megalin was responsible for the endocytosis of DBP, we applied a well-established antagonist to inhibit megalin activity in the tubules. The receptor-associated protein (RAP) is a 39 kDa protein that specifically prevents binding of ligands to LDL receptor–related receptors and that has been used previously to block megalin activity in vivo. In these studies, RAP did not affect other known endocytic receptors in proximal tubular cells (Moestrup et al., 1995, 1996). By incubation of wild-type and knockout kidney sections with radiolabeled RAP, megalin was found to be the only RAP-binding protein in the kidney (Figures 4B and 4C). When ^{125}I-labeled DBP was microinfused into proximal tubules in the presence of RAP, only 8.2% of the ligand was taken up (Table 1), whereas the remainder was excreted into the urine. Receptor-mediated uptake and lysosomal degradation of ^{125}I-DBP was also confirmed in cultured BN16 choriocarcinoma cells expressing megalin. In these cells, 18% ± 0.01% of the added ^{125}I-DBP was degraded within 4 hr. Degradation was inhibited significantly by RAP (3.9% ± 0.01%) or chloroquine (8.3% ± 0.01%). Combined application of both inhibitors had no additive effect (2.4% ± 0.1% ligand degraded in 4 hr).

Given the ability of megalin to clear DBP from the glomerular filtrate, we next tested whether DBP and 25-(OH) vitamin D_3/DBP complexes are continuously filtered through the glomerulus and reabsorbed by this receptor in the proximal tubules. ^{125}I-labeled DBP was infused directly into the renal arteries of rats, and the uptake of radioactivity in kidney, blood, and urine was determined (see Experimental Procedures). Sixty minutes after infusion, 86.5% of the radioactivity was found in the circulation as proteolytic degradation products (Table 2). In contrast, when megalin activity was blocked

Table 2. Uptake of ^{125}I-DBP and ^{3}H-25-(OH) Vitamin D_3/DBP into Perfused Rat Kidneys

			Percent Recovered Ligand (± SEM)		
Ligand	t (Min)	n	Urine	Kidney	Blood
^{125}I-DBP	60	5	7.6 ± 3.3	5.8 ± 2.4	86.5 ± 5.9
^{125}I-DBP + RAP	60	4	84.2 ± 7.6	2.4 ± 2.0	13.4 ± 7.8
^{3}H-25-(OH) D_3/DBP	60	5	7.4 ± 6.7	7.8 ± 4.6	84.6 ± 6.2
^{3}H-25-(OH) D_3/DBP + RAP	10	1	6.8	0.6	92.6
	30	2	41.7 ± 11	3.8 ± 0.6	54.5 ± 10.2
	60	4	86.1 ± 11	0.05 ± 0.02	13.9 ± 11.2

Percent of total radioactivity recovered in blood, kidney, and urine at the indicated time points after injection of the ligand is shown. For details, see Experimental Procedures.
n, number of animals; SEM, standard error of the mean; t, perfusion time in minutes.

by coinjection of RAP, only 13.4% of ^{125}I-DBP was detected in the plasma, while 84.2% was excreted into the urine. A similar experiment was performed using complexes of ^{3}H-25-(OH) vitamin D_3 and unlabeled DBP. To follow the kinetics of glomerular filtration, the ligand was allowed one pass (10 min perfusion) or several passes (30 or 60 min perfusion) through the kidney (see Experimental Procedures). Like ^{125}I-DBP, ^{3}H-25-(OH) vitamin D_3 also appeared in the urine when megalin function was blocked by coinfusion of RAP (Table 2). The amount of the vitamin excreted correlated directly with the time of perfusion. Thus, 6.8% of the tracer was filtered per passage through the kidney. After continued perfusion for 60 min, virtually all ^{3}H-25-(OH) vitamin D_3 was recovered in the urine (86.1%). When ^{3}H-25-(OH) vitamin D_3 was infused in the absence of RAP, 84.6% of the radioactivity was still present in the circulation after 60 min of perfusion. Taken together, these findings indicated that megalin retrieves DBP and 25-(OH) vitamin D_3/DBP complexes from the glomerular filtrate.

Prompted by the efficient megalin-mediated uptake of 25-(OH) vitamin D_3/DBP complexes, we analyzed whether this pathway also provided 25-(OH) vitamin D_3 for hydroxylation. To test for conversion, ^{3}H-labeled vitamin D_3 metabolites were recovered from blood and urine of perfused animals (as in Table 2) and analyzed by high pressure liquid chromatography (HPLC). To distinguish between precursors taken up from the glomerular filtrate (luminal) or the circulation (basolateral), we used rats that had been perfused with ^{3}H-25-(OH) D_3/DBP in the presence or absence of RAP for 30 to 40 min. At this time point, approximately 40% of ^{3}H-25-(OH) vitamin D_3 has been filtered and reabsorbed by the proximal tubules. In the presence of RAP, the filtered precursor molecules were lost in the urine and only nonfiltered metabolites were available for conversion (Table 2). As depicted in Figure 5B, the vitamin D_3 metabolites recovered from the circulation of animals infused with tracer only were present as ^{3}H-25-(OH) vitamin D_3 as well as ^{3}H-1,25 $(OH)_2$ vitamin D_3. In contrast, only ^{3}H-25-(OH) vitamin D_3, and no conversion products, were identified in plasma or urine of animals coinfused with RAP (Figure 5C). These findings demonstrate that filtered 25-(OH) vitamin D_3 molecules are hydroxylated and that inactivation of megalin prevents delivery of the precursor to tubular cells.

Vitamin D Deficiency and Bone Formation Defects in Megalin$^{-/-}$ Mice

Megalin knockout mice excrete massive amounts of DBP in the urine (Figure 2). Because 1% of DBP molecules in the plasma are found in complex with 25-(OH) vitamin D_3, we reasoned that these animals also lose significant amounts of the vitamin. We therefore investigated the consequence of megalin deficiency for vitamin D homeostasis. Western blot analysis demonstrated that DBP levels in the circulation were similar in wild-type and in megalin$^{-/-}$ mice, demonstrating that the plasma half-life of the protein was unchanged regardless if it was turned over by tubular degradation in wild-type mice or lost in the urine of knockout mice (Figure 6). However, urinary excretion of DBP in receptor-deficient animals resulted in the concomitant excretion of 25-(OH) vitamin D_3, which was not present in control urine (Figure 6). It was calculated that the mice lose 0.3–0.4 mg DBP and 4–5 ng 25-(OH) vitamin D_3 every 24 hr (see Experimental Procedures for details). Most importantly, urinary loss of the vitamin coincided with an 80% reduction in plasma 25-(OH) vitamin D_3 levels (Figure 6). Because plasma levels of 25-(OH) vitamin D_3 are an indicator of bioavailability of the vitamin, this suggested a status of vitamin D deficiency in megalin$^{-/-}$ animals. Similar to patients and animal models with vitamin D deficiencies, megalin$^{-/-}$ mice were characterized by severe bone formation defects. The mice were significantly retarded in growth (Figures 1A and 1B) and exhibited a dramatic reduction in density of the bones, as shown by contact X-ray imaging (Figures 7A and 7B). Histological evaluation of the vertebral bodies revealed highly irregular and scalloped bone surfaces compared to control animals (Figures 7C and 7D). The lack of continuity of the bone surfaces in the knockout mice was most likely a consequence of perforating osteoclastic resorptions occurring in high bone turnover conditions. Finally, disturbances in bone metabolism were confirmed by a 3.5-fold elevation in mean serum alkaline phosphatase levels (352 vs 106 U/l) and by increased concentrations of hydroxyproline in the urine (417 vs 193 μmol/l) as compared to control mice.

Discussion

Our findings demonstrate that complexes of 25-(OH) vitamin D_3 and DBP are filtered through the glomerulus

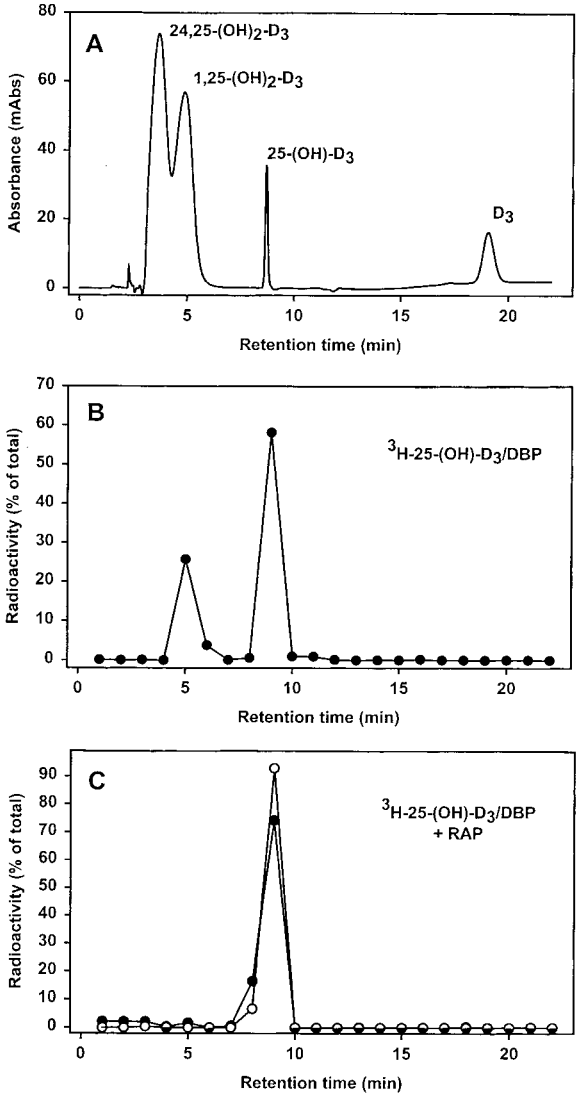

Figure 5. HPLC Analysis of Vitamin D_3 Metabolites in Rat Plasma and Urine

Vitamin D_3 metabolites were purified from plasma and urine samples of rats infused with ^{3}H-25-(OH) D_3/DBP (B) or ^{3}H-25-(OH) D_3/DBP + RAP (C) and subjected to high pressure liquid chromatography (HPLC) analysis on C_{18} reverse-phased column (see Experimental Procedures). Radioactivity recovered in each fraction was expressed as the percentage of total radioactivity recovered (33,000 cpm in [B], 40,000–48,000 cpm in [C]). Representative profiles of individual animals of each group (six animals per group) are shown. The percent of radioactivity in the 1,25-$(OH)_2$ vitamin D_3 peak (B) varied between 10% (30 min perfusion) and 25% (45 min perfusion). Closed circles indicate blood; open circles indicate urine samples. For comparison, the HPLC profile of standard vitamin D_3 metabolites is shown in (A). D_3, vitamin D_3.

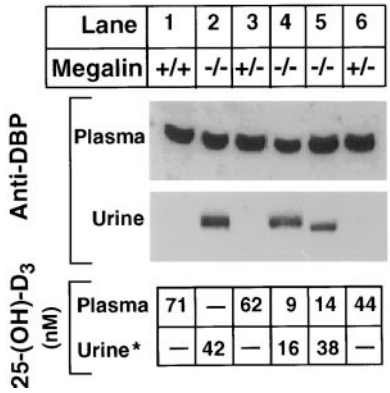

Figure 6. Quantification of DBP and 25-(OH) Vitamin D_3 in Mouse Plasma and Urine

Fifteen microliters of urine and 0.5 μl of plasma obtained from mice of the indicated megalin genotypes were subjected to 10% nonreducing SDS-PAGE and immunoblot analysis using anti-DBP antiserum and ECL. 25-(OH) vitamin D_3 was measured in parallel plasma and urine samples as described in Experimental Procedures. Where no number is given, the measurement was below the detection limit of the assay (<5 nM). *, urine values were obtained on samples concentrated 30-fold by freeze-drying.

and reabsorbed in the proximal tubules by the endocytic receptor megalin. 25-(OH) vitamin D_3 molecules taken up via this receptor pathway are converted into 1,25-$(OH)_2$ vitamin D_3. Malabsorption of 25-(OH) vitamin D_3/DBP complexes in megalin knockout mice results in severe vitamin D deficiency and bone formation defects.

Previously, the role of DBP in the delivery of the 25-(OH) vitamin D_3 to the kidney was unclear. In established proximal tubular cell lines, uptake of 25-(OH) vitamin D_3 was observed when the steroid was added in free form, while little uptake was seen with complexes of 25-(OH) vitamin D_3/DBP (Keenan and Holmes, 1991). Therefore, it was hypothesized that in vivo uptake of the precursor also proceeds by diffusion of the free steroid through the plasma membrane. DBP was believed to solely regulate the amount of free 25-(OH) vitamin D_3 available in the circulation (Mendel, 1989; Haddad, 1995). This hypothesis was confounded by the observation that only 0.003% of 25-(OH) vitamin D_3 in the circulation is present in the free form (0.6 pg/ml plasma), whereas the rest is bound to DBP or serum albumin (20 ng/ml plasma) (Bikle et al., 1986). At the same time, approximately 0.3 to 0.5 ng of 25-(OH) vitamin D_3 are converted into 1, 25-$(OH)_2$ vitamin D_3 by the kidney every day (Bikle et al., 1985). The requirement for such massive intake of precursor strongly argues for additional steroid uptake pathways whereby the kidney gains access to the pool of bound 25-(OH) vitamin D_3. Based on our findings, it is likely that megalin-mediated endocytosis constitutes the pathway for delivery of the complexed precursor. Megalin knockout mice lose approximately 4–5 ng 25-(OH) vitamin D_3 in the urine over 24 hr (based on data in Figure 6). In wild-type animals, the same amount of 25-(OH) vitamin D_3 is taken up into epithelial cells via this receptor pathway, corresponding to ten times the amount of precursor metabolized per day. Besides significant efficiency, megalin-mediated uptake would also ensure cell type specificity for the delivery of the precursor. Because established proximal tubular cell lines (e.g., LLC-PK1) express very little megalin (E. I. C. et al., unpublished observations), this receptor pathway may have been missed in previous studies.

When megalin activity was blocked by RAP, no conversion products were recovered from rats infused with ^{3}H-25-(OH) vitamin D_3 (Figure 5C). This result indicated

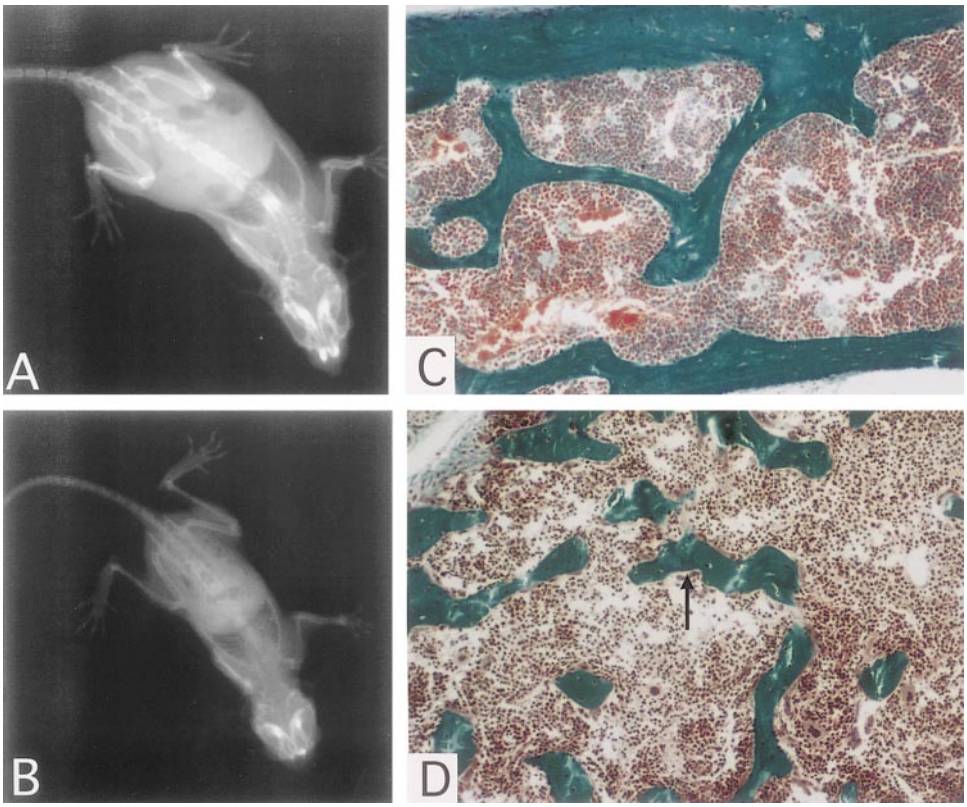

Figure 7. Bone Structure of Wild-Type and Megalin$^{-/-}$ Mice

(A and B) Radiographic imaging of wild-type (A) and megalin$^{-/-}$ littermates at 6 weeks of age (B).
(C and D) Histological analysis of the vertebral bodies of wild-type (C) and megalin$^{-/-}$ mice (D). Undecalcified Goldner-stained sections were produced from the vertebral bodies. The arrow denotes irregular bone surfaces due to osteoclastic resorptions in the megalin-deficient mouse (D) compared to smooth connected cancellous bone surfaces in the control animal (C).

that receptor-mediated uptake from the lumen of the proximal tubule delivers precursors for hydroxylation. Such a role of tubular resorption processes in vitamin D metabolism is supported by studies in patients with renal defects. Similar to megalin knockout mice, patients that suffer from tubular resorption deficiency (Fanconi syndrome) exhibit vitamin D deficiency and bone disease (rickets, osteomalacia) (Harrison, 1953). Fanconi syndrome is caused by various genetic as well as environmental factors (e.g., heavy metal poisoning) and is characterized by an inability of the proximal tubules to reabsorb filtered macromolecules. One of the proteins excreted in the urine of Fanconi patients is DBP (Teranishi et al., 1983; Figure 3 inset). Therefore, the disturbances in calcium and bone metabolism in these patients might be explained in part by the urinary loss of 25-(OH) vitamin D_3 bound to the carrier. In addition, St. John et al. (1992) showed that in individuals with various degrees of renal failure, the glomerular filtration rate was directly correlated with the plasma concentrations of 1,25-$(OH)_2$ vitamin D_3. This finding suggests that glomerular filtration is a prerequisite for the generation of 1,25-$(OH)_2$ vitamin D_3.

Our results demonstrate that megalin constitutes a highly efficient pathway for delivery of 25-(OH) vitamin D_3 to tubular epithelial cells. These findings do not exclude other pathways for delivery of the precursor, such as uptake of the free steroid from the circulation. The limited number ($n = 8$) and the small size of megalin$^{-/-}$ animals precluded extensive characterization of electrolyte and endocrine parameters [e.g., parathyroid hormone and 1, 25-$(OH)_2$ vitamin D_3] to answer this question. Nevertheless, phenotypic analysis of mice genetically deficient for DBP strongly supports a role of megalin and its ligand DBP in the renal vitamin D_3 metabolism. In DBP$^{-/-}$ mice, the plasma half-life of 25-(OH) vitamin D_3 is markedly reduced and urinary loss of the metabolite increased. This imbalance results in low plasma levels of 25-(OH) vitamin D_3 and 1,25-$(OH)_2$ vitamin D_3 and, as a consequence, in defects of bone metabolism (Cooke et al., 1997).

The present study identifies a pathway for renal uptake and activation of 25-(OH) vitamin D_3 involving the endocytic receptor megalin. Such a role of tubular endocytic processes in vitamin D homeostasis has not been known previously. We propose a model in which megalin mediates the tubular uptake of the steroid-carrier complexes filtered through the glomerulus (Figure 8). The carrier DBP is degraded in lysosomes, while 25-(OH) vitamin D_3 is converted into 1,25-$(OH)_2$ vitamin D_3 and resecreted into the circulation. This pathway exhibits striking resemblance to the cellular uptake of LDL by the LDL receptor. Both receptors mediate the endocytosis of lipid-carrier complexes by binding the apoprotein

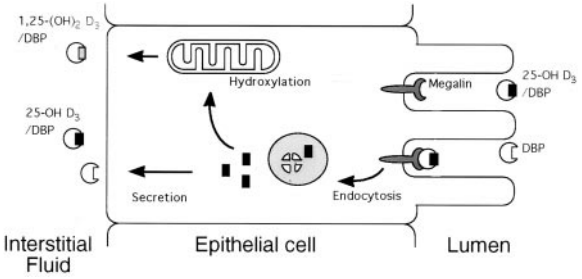

Figure 8. Model of Megalin Function in Renal Uptake and Activation of 25-(OH) Vitamin D_3

DBP and 25-(OH) vitamin D_3/DBP complexes are filtered through the glomerulus and endocytosed into the proximal tubular epithelium via megalin. The complexes are delivered to lysosomal compartments, where DBP is degraded and the vitamin released into the cytosol. 25-(OH) vitamin D_3 molecules are either secreted directly or hydroxylated to 1,25 $(OH)_2$ vitamin D_3 prior to release into the interstitial fluid. There, they are complexed by free DBP molecules.

moieties (apolipoprotein B-100 or DBP). The apoproteins are degraded while the steroids [cholesterol or 25-(OH) vitamin D_3] are delivered to the intracellular metabolism.

Similar to vitamin D metabolites, other steroid hormones are also transported in the circulation by specific plasma carrier proteins. These include sex hormone–binding globulin and corticosteroid-binding globulin. As suggested for DBP, these carriers are believed to keep steroids in a biologically inactive state and regulate the plasma concentration of free hormones that enter cells by diffusion (Siiteri and Simberg, 1986; Mendel, 1989). Such a concept requires nonspecific entry of steroids into all cells of an organism in order to find their intracellular hormone receptors. In contrast, receptor-mediated endocytosis of steroid-carrier complexes would offer the advantage of cell type–specific delivery. The existence of such uptake pathways for bound steroids was proposed because metabolic clearance rates were better correlated with the total than the free fraction of the hormones, indicating uptake of both free and bound steroids (Siiteri et al., 1982). Furthermore, steroid target tissues have been shown to express binding sites for carrier proteins (reviews in Siiteri and Simberg, 1986; Porto et al., 1995). Finally, endocytosis of testosterone-estradiol-binding globulin was demonstrated directly in the human breast cancer cell line MCF-7 (Porto et al., 1991). So far, the physiological significance of these endocytic pathways for steroid hormone action remained unclear. We now report one example for the biological importance of such endocytic pathways (megalin) inasmuch as they preserve plasma vitamin D levels and deliver to the kidney the precursor for generation of active 1,25-$(OH)_2$ vitamin D_3. Clearly, the resorption of filtered vitamin D metabolites in the renal proximal tubule is unique, as it requires especially efficient uptake pathways to prevent urinary loss of the vitamins. Nevertheless, it is intriguing to speculate that similar endocytic mechanisms may exist in other tissues. If confirmed for other hormonal systems, these findings not only have elucidated a central step in the vitamin D metabolism but may change current concepts in steroid hormone metabolism reaching far beyond systemic calcium and bone metabolism.

Experimental Procedures

Materials and Sample Collection

Urine samples from patients with myeloma-associated Fanconi syndrome were kindly provided by P. Aucouturier (Hôpital de Paris), vitamin D_3 metabolites by A.-M. Kissmeyer (Leo Pharmaceutical, Copenhagen), anti-aquaporin IgG by S. Nielsen (University of Aarhus, Aarhus) and M. Knepper (NIH, Bethesda), anti-megalin antiserum by P. Verroust (Hôpital Tenon, Paris), and purified megalin by S. Moestrup (University of Aarhus, Aarhus). Anti-human DBP antiserum was purchased from DAKO (Hamburg), ^{3}H-25-(OH) vitamin D_3 from Amersham (Braunschweig). Sterol/DBP complexes were prepared by incubating purified DBP with a 100-fold molar excess of ^{3}H-25-(OH) vitamin D_3 for 16 hr at 4°C under N_2 in the dark. Mouse plasma was collected by retroorbital bleeding. For urine collection, mice were placed in metabolic cages for 16 hr and given 10% sucrose in drinking water. Urine samples obtained (approximately 8 ml/24 hr) were qualitatively indistinguishable from samples collected without sucrose load. Urine volume per hour and creatinine levels (~6 mg/dl) were identical in megalin$^{-/-}$ and in control mice (not shown). Concentrations of DBP and 25-(OH) vitamin D_3 in plasma and urine were determined by semiquantitative Western blot analysis (for DBP) or competitive protein binding assay (for 25-(OH) vitamin D_3; Immundiagnostik, Bensheim).

Receptor Binding Assay

Binding of DBP to megalin was quantified by BIAcore (Biosensor, Sweden) as described previously (Moestrup et al., 1998). A continuous flow of HBS buffer (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.005% surfactant P20 [pH 7.4]) passing over the sensor surface was maintained at 5 μl/min. The carboxylated dextran matrix of the sensor chip flow cell was activated by injection of 60 μl of a solution containing 0.2 M N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccimide in water. Then, 180 μl of 10 mM sodium acetate (pH 4.5) containing 10 μg/ml purified rabbit megalin was injected. The remaining binding sites were blocked by subsequent injection of 35 μl 1 M ethanolamine (pH 8.5). The surface plasmon resonance signal from immobilized megalin generated 22,000 BIAcore response units (RU) equivalent to 34 fmol megalin/mm^2. To test DBP binding, rabbit megalin immobilized on CM5 sensor chip was incubated with 30 μg/ml human DBP in 10 mM HEPES, 150 mM NaCl, 1.5 mM $CaCl_2$, 1 mM EGTA (pH 7.4) or in 10 mM HEPES, 150 mM NaCl, 20 mM EDTA (pH 7.4), and the relative increase in response between megalin and control flow channels was determined. Kinetic parameters were determined by using the BIAevaluation 3.0 software.

Histological Analysis

The preparation of light and electron microscopical sections of kidney tissues has been described previously (Christensen et al., 1995). For immunolabeling, the sections were incubated with primary antibodies either at room temperature for 1 hr or overnight at 4°C, followed by incubation with peroxidase-conjugated secondary antibody and detection with diaminobenzidine. Binding of DBP and ^{125}I-RAP to kidney sections was carried out as published (Christensen et al., 1995). Briefly, the sections were incubated for 30 min with phosphate buffer containing 100 mM DBP or 1 μCi/ml ^{125}I-RAP. Bound DBP was detected by peroxidase-conjugated anti-DBP antibody and diaminobenzidine. For detection of ^{125}I-RAP, electron micrographs were subjected to autoradiography (Christensen et al., 1995).

Kidney Uptake Experiments

Microinfusion assays of proximal convoluted tubules of anesthetized Wistar rats have been described previously (Moestrup et al., 1995, 1996). A total of 0.5–2.7 pmol ^{125}I-DBP (in 50 nl) were infused into individual proximal tubules for 2 min in the presence or absence of 0.4 mg/ml RAP. Subsequently, urine was collected from a urether catheter for 30 min. Tubular uptake was calculated as the difference

between trichloroacetic acid–precipitable counts microinfused versus recovered in urine. Perfusion of total rat kidneys was performed as follows: male Lewis rats fed a diet low in vitamin D and calcium (EF R/M 10 mm, Sniff, Soest) for 6 weeks were anesthetized and infused with 1% bovine serum albumin in 0.9% NaCl through the external jugular vein to enhance diuresis. The right kidney was clamped, and the aorta was occluded proximal and distal to the left renal artery. Subsequently, the left kidney was perfused for 10 min through a catheter placed in the aorta between the two occlusion sites with 1 ml of saline containing 50 pM ^{125}I-DBP or 12 ng/ml ^{3}H-25-(OH) vitamin D_3 complexed with DBP. Where indicated, the infusion also contained 1 mg/ml RAP. Under these conditions, the tracer was allowed one pass through the glomerulus (10 min perfusion time). For extended filtration, the aorta was repaired and arterial blood flow through the kidney restored for 30 or 60 min by opening the proximal occlusion site. Therefore, the entire arterial blood flow was directed through the kidney. During the perfusion, urine was collected from the left urether. At the end of the experiment, blood was collected from the renal vein effluent, and the total amount of radioactivity in urine, blood, and kidney was determined. On average, 60% to 80% of total radioactivity injected was recovered. To isolate tritium-labeled vitamin D_3 metabolites, the urine and blood samples were precipitated with 66% acetonitrile. The supernatant was diluted to 15% acetonitrile and applied to activated C18 cartridges (SePak, Waters, Milford). The cartridges were washed with 3 ml of 70% methanol and 10 ml H_2O. The vitamin metabolites were then eluted in 4 ml acetonitrile and dried under constant nitrogen flow. Subsequently, the samples were resuspended in 100 μl isopropanol and chromatographed on C_{18} reverse-phased column (VYDAC, #218TP104) in 85% methanol, 15% H_2O as published (Omdahl et al., 1980). Radioactivity was measured in all fractions and expressed as the percentage of total radioactivity recovered. For comparison, the retention profiles of unlabeled and tritium-labeled vitamin D_3 metabolites were determined.

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