The kidney in vitamin B\textsubscript{12} and folate homeostasis: characterization of receptors for tubular uptake of vitamins and carrier proteins

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Birn, Henrik. The kidney in vitamin B\textsubscript{12} and folate homeostasis: characterization of receptors for tubular uptake of vitamins and carrier proteins. Am J Physiol Renal Physiol 291: F22–F36, 2006; doi:10.1152/ajprenal.00385.2005.—Over the past 10 years, animal studies have uncovered the molecular mechanisms for the renal tubular recovery of filtered vitamin and vitamin carrier proteins. Relatively few endocytic receptors are responsible for the proximal tubule uptake of a number of different vitamins, preventing urinary losses. In addition to vitamin conservation, tubular uptake by endocytosis is important to vitamin metabolism and homeostasis. The present review focuses on the receptors involved in renal tubular recovery of folate, vitamin B\textsubscript{12}, and their carrier proteins. The multiligand receptor megalin is important for the uptake and tubular accumulation of vitamin B\textsubscript{12}. During vitamin load, the kidney accumulates large amounts of free vitamin B\textsubscript{12}, suggesting a possible storage function. In addition, vitamin B\textsubscript{12} is metabolized in the kidney, suggesting a role in vitamin homeostasis. The folate receptor is important for the conservation of folate, mediating endocytosis of the vitamin. Interaction between the structurally closely related, soluble folate-binding protein and megalin suggests that megalin plays an additional role in the uptake of folate bound to filtered folate-binding protein. A third endocytic receptor, the intrinsic factor-B\textsubscript{12} receptor cubilin-amnionless complex, is essential to the renal tubular uptake of albumin, a carrier of folate. In conclusion, uptake is mediated by interaction with specific endocytic receptors also involved in the renal uptake of other vitamins and vitamin carriers. Little is known about the mechanisms regulating intracellular transport and release of vitamins, and whereas tubular uptake is a constitutive process, this may be regulated, e.g., by vitamin status.

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megalin; cubilin; folate-binding protein; proximal tubule; endocytosis

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The kidney is an excretory organ, in which serum constituents are specifically sorted either for urinary excretion or for conservation within the body. During this process, the kidneys must handle a large number of different endo- and exogenous substances, many of which are essential to life. Vitamins constitute such important substances normally required from food sources and essential to fundamental biological reactions. Vitamin B\textsubscript{12} and folate both belong to the group of water-soluble B vitamins. While structurally different, they interact in common biochemical reactions and reveal a very similar picture of megaloblastic anemia when deficient. They are present in serum either free or bound to carrier proteins and are filtered in the renal glomeruli. Estimations of the filtration fraction suggest that efficient renal tubular reabsorption of both is essential to prevent excessive urinary losses in both humans and animals. Animal studies within recent years have established close interaction and a functional relationship between the structurally very different receptors involved in the renal tubular uptake of these vitamins and their carrier proteins. The present review will focus on the receptors and molecular mechanism mediating renal uptake of folate and B\textsubscript{12} and of their carrier proteins. The common features of these processes, involved also in the kidney uptake of a number of other vitamins, hormones, and carrier proteins, will be discussed.

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FOLATE

Folate is a water-soluble vitamin recognized in the 1930s as a hematopoietic factor present in liver and yeast extracts. It was isolated from 4 tons of spinach leaves in 1941 and reported to be concentrated in liver and kidney. Chemically, it is made of a pteridine core ring linked to \textit{p}-aminobenzoic acid. Additional glutamate residues are attached to the \textit{p}-aminobenzoic acid, forming mono- or polyglutamate forms. Mammals are able to synthesize the pteridine ring but are unable to couple it to other compounds and are thus dependent on either dietary intake or bacterial synthesis within the intestine. The term folic acid generally used for the synthetic pteroylglutamic acid representing fully oxidized folate. The biologically active form is reduced tetrahydrofolate (THF)\textsuperscript{1}, serving as an essential cofactor in methylation reactions, including the vitamin B\textsubscript{12}-dependent formation of methionine from homocysteine, and as a carrier of one-carbon units involved in the synthesis of purines and pyrimidines (Fig. 1). Whereas intracellular folate accumulates in the polyglutamate form, folate in serum is in the monoglutamate form, the only form that is transported actively.

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\textsuperscript{1} The present review will apply the term “folate” when referring to all different forms of the vitamin that may be converted into the active form.

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across cell membranes. Folate deficiency is associated with homocysteinemia, megaloblastic anemia, leuco- and thrombocytopenia, cardiovascular disease, embryonic defects, in particular neural tube defects, and, possibly, malignancies (135).

Dietary folate is found in vegetables, fruits, grain, yeast, and dairy products. Total body content of folate has been estimated to be 38–96 mg (86–165 μmol), being slowly catabolized or excreted mainly by the fecal route.

Following ingestion, folate polyglutamate is converted into monoglutamate by intestinal brush-border glutamylcarboxylase and absorbed most likely by a carrier-mediated mechanism involving the reduced folate carrier (RFC) (86). The dominant folate form in serum is 5-methyltetrahydrofolate (5-MTHF), present either free, bound to high-affinity folate-binding protein (FBP), or loosely associated with other serum proteins including albumin (61, 132). The fraction of folate in serum may be increased by methylation of enzyme-bound B12, serves as a cofactor for cytosolic methionine synthase. This enzyme transfers a methyl group from 5-MTHF to homocysteine, forming methionine, the single known metabolic pathway common to folate and B12 (blue arrows). The transfer of a methyl to homocysteine causes reduction of methionine synthase-bound B12 and is followed by remethylation using a methyl group donated by 5-MTHF. Methionine may be converted into adenosyl-methionine, an important methyl donor in a number of different reactions. It is clear from the diagram why both folate and vitamin B12 deficiency may lead to elevated homocysteine due to inhibited conversion of this into methionine. Vitamin B12 deficiency may also lead to elevated methylnalonic acid (MMA) as a result of accumulation of methylnalonic-CoA which is converted to MMA. Because the conversion of 5,10-methylene-THF to 5-MTHF is essentially irreversible, folate accumulates as 5-MTHF when the methionine synthase is blocked due to B12 deficiency. This results in a functional folate deficiency despite elevated 5-MTHF-levels. Thus both folate and vitamin B12 deficiency may lead to inhibited synthesis of nucleotides for RNA and DNA, causing megaloblastic anemia. Vitamin B12 deficiency is also associated with neurological disturbances, which may be explained by decreased methylation of myelin basic protein, an important constituent of myelin, due to interruption of methionine formation. In contrast, neurological symptoms are normally not associated with folate deficiency. This may be explained, in part, by the ability of nerve tissue to concentrate folate significantly above serum levels. Enzymes appear in italics. Vitamin-B12 as an enzyme-bound cofactor is indicated in red. Dotted arrows indicate that the processes may involve several transfer and/or biochemical steps not shown. The processes are reviewed in Refs. 25, 110, and 123. DHF, dihydrofolate.
VITAMIN B12

Vitamin B12 was originally identified as the antianemic, extrinsic factor present in liver and liver extract, reversing the classic symptoms of megaloblastic anemia. In 1948, vitamin B12 was isolated from liver as a red crystalline substance causing clinical remission in cases of pernicious anemia. Chemically, it contains a corrin ring consisting of four reduced pyrrole rings surrounding a central cobalt atom. The different chemical groups attached to the central cobalt atom classify the type of cobalamins as vitamin B12 or B12. These include the coenzyme forms methylcobalamin (MeB12) and 5-deoxyadenosylcobalamin, as well as hydroxycobalamin (HOx), folates, vitamin B12 into the active forms, methylcobalamin and 5-deoxyadenosylcobalamin. The function of HC in adults is not fully understood, although a role in the clearance of cobalamin analogs from the circulation has been suggested (39). Vitamin B12 is a highly conserved vitamin, and the daily losses in humans is estimated to be 0.1–0.2% of total body content (1). The highest losses occur through feces, and B12 is secreted in bile, although to a large extent reabsorbed when supported by sufficient secretion of IF and a functional ileal-absorptive apparatus (48).

FOLATE, VITAMIN B12, AND THE KIDNEY

The renal uptake of both folate and vitamin B12 involves glomerular filtration followed by tubular reabsorption. Significant amounts of vitamins are filtered daily, and because urinary excretion of B12 and intact folate is low, both are reabsorbed within the renal tubular system to prevent urinary loss. Furthermore, tubular uptake may result in kidney accumulation and possibly metabolism of B12. In addition to the physiological relevance, interest in the molecular mechanisms regulating these processes is stimulated by the clinical focus on folate and vitamin B12 in relation to renal disease. Epidemiological studies show an association between elevated levels of homocysteine and an increased risk of cardiovascular disease (25) also in renal patients (5, 32). Folate supplementation is able to reduce elevated homocysteine levels in renal patients (32), and it was suggested that additional supplementation with vitamin B12 may further lower homocysteine levels even in patients with normal serum B12 levels (37). The metabolic changes underlying elevated homocysteine levels in renal disease are not fully understood; however, the use of high-dose folate and/or vitamin B12 supplementation in renal patients attracts interest to the way these vitamins are handled by the kidney.

Glomerular Filtration of Vitamins and Carrier Proteins

The molecular mass of folate (∼440 Da) suggests that free folate is freely filtered in the glomeruli. Due to the association of folate with serum proteins, the filtration of folate in humans is estimated to be 50–65% of that of inulin (46), suggesting a filtered load of free folate of ∼1 mg/24 h in humans (127). A

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2 The term “vitamin B12” is chemically restricted to cyanocobalamin; however, the present review will refer to all potential biologically active cobalamins as vitamin B12 or B12. These include the coenzyme forms methylcobalamin and 5-deoxyadenosylcobalamin, as well as hydroxycobalamin and cyanocobalamin, all found in serum with methylcobalamin as the dominant form (45).
small fraction of folate is bound to serum FBP. The molecular mass of $\sim$35 kDa suggests that this protein to a large extent is filtered, and FBP has been detected in human urine at a concentration of 0.5–4 nM (50). Most protein-bound folate in serum is loosely associated with albumin (132). The fraction of filtered albumin is traditionally considered to be low; however, recent evidence has suggested that a much larger amount is filtered in the normal kidney (117), although this is yet to be established.

The glomerular filtration of vitamin B$_{12}$ similarly is dependent on serum protein binding and thus on the concentration of B$_{12}$ in serum. In humans, no urinary excretion of B$_{12}$ was detected at concentrations $<1.1$ ng/ml ($\sim$800 pM), whereas at concentrations $>12$ ng/ml ($\sim$8 nM) B$_{12}$ is excreted at a rate similar to the glomerular filtration rate (147). Thus unbound B$_{12}$ is freely filtered and may in fact serve as a marker to estimate glomerular filtration rate (99). At normal serum B$_{12}$ concentration, the filtered load of the vitamin in humans has been estimated to be 1.5 $\mu$g (79). The molecular mass of TC suggests that the TC-B$_{12}$ complex is filtered, supported by the demonstration of small amounts of B$_{12}$-binding proteins, including TC, in human urine (13, 79, 143). Most circulating HC, originating from myeloid cells, is heavily glycosylated (39), probably limiting filtration of this protein, and the origin of urinary HC remains to be established.

**Renal Tubular Reabsorption of Filtered Vitamin and Carrier Proteins**

The major pathway for the uptake of filtered macromolecules in the proximal tubules is by receptor-mediated endocytosis. This involves the specific binding of a ligand to a receptor in the apical plasma membrane. The receptor–ligand complex is internalized by invagination of the plasma membrane caused by adaptor molecule-mediated formation of a cytoplasmic clathrin coat (122). Internalization is followed by dissociation of the invaginations from the plasma membrane, forming vesicles. While the coat detaches, vesicles may fuse with other newly formed vesicles, or with an existing pool of larger vesicles, followed by acidification of the intravesicular lumen and the dissociation of the ligand from the receptor. The ligand may be further transported into lysosomes for degradation, or possibly storage, or into the cytosol for further processing/transport. The receptor is recycled back to the luminal membranes through a recycling compartment; however, it may also be transported to lysosomes for degradation. In addition to the formation of a clathrin coat, other mechanisms of internalization have been established, including noncoated endocytosis and internalization by caveolae involving the protein caveolin (63). It has been proposed that glycosylphosphatidylinositol (GPI)-anchored proteins may be internalized by caveolae rather than by clathrin-coated pits (3).

Several different receptors mediating endocytosis of filtered ligands have been identified in the kidney proximal tubule cell (reviewed in Ref. 28). The receptors, megalin and cubilin, and the folate receptor (FR) have been implicated in the uptake of folate, vitamin B$_{12}$, and their carrier proteins. The structure, renal expression, regulation, and mutual interaction of these receptors will be reviewed, followed by a discussion of their possible role in the renal handling of B$_{12}$ and folate.

**Megalin**. Megalin is a multifunctional, endocytic receptor binding a number of structurally and functionally different

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<th>Table 1. <strong>Ligands for megalin and cubilin</strong></th>
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<td><strong>Hormones, hormone precursors, and signaling proteins</strong></td>
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<td>Hedgehog protein</td>
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<td>Angiotensin II</td>
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<td>Leptin</td>
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<td>Bone morphogenic protein 4</td>
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<td>Ca$^{2+}$</td>
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<tr>
<td>Cytochrome c</td>
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<td>Receptor for seminal vesicle secretory protein II</td>
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AMN, amnionless.
ligands (Table 1). Ligands for megalin that may be filtered in the glomeruli include vitamin D-binding protein (DBP), retinol-binding protein (RBP), TC-B12, FBP, parathyroid hormone, insulin, epidermal growth factor (EGF), prolactin, albumin, hemoglobin, myoglobin, β2- and α1-microglobulin, apolipoprotein H, lysozyme, cytochrome c, and α-amylase.

**STRUCTURE.** Megalin was originally identified as the antigen in Heymann nephritis, a rat model of membranous glomerulonephritis (Fig. 2) (69). It is a 600-kDa protein (Fig. 3) with a large NH2-terminal extracellular domain, a single transmembrane domain, and a short COOH-terminal cytoplasmic tail (57, 98, 120). The protein belongs to the LDL receptor family, sharing common features with, in particular, the LDL receptor-related protein (LRP). The extracellular domain is composed of four clusters of cysteine-rich complement-type/LDL receptor class A repeats separated by 17 EGF-like repeats. The latter contain YWTD motifs involved in pH-dependent release of ligands (36). Megalin binds Ca2+ very strongly, constituting ~40% of all Ca2+-binding activity in the renal cortex (30). The ligand-binding type A repeats are negatively charged Ca2+-binding protein domains, and Ca2+ is important for most ligand binding to megalin. An important role of megalin in Ca2+ metabolism has also been proposed (27, 57, 151). Site-directed mutations of basic amino acid residues in aprotinin, a 6-kDa proteinase inhibitor and a ligand for megalin, decrease the affinity for the receptor, suggesting that binding is charge dependent and favored by cationic sites on the ligands (91). However, many ligands are anionic proteins, indicating that the distribution of charge rather than the overall isoelectric point is important for binding.

**RENAL EXPRESSION.** Megalin is heavily expressed in the kidney proximal tubule brush border and all components of the luminal endocytic apparatus (Fig. 4) (26, 30, 69). In addition, smaller amounts of immunoreactive megalin have been identified in lysosomal structures. Megalin has also been identified in the glomerular podocytes of Lewis rat kidney (70). In addition to the kidney, megalin is expressed in a number of other epithelia (reviewed in Ref. 27), including the ileum (11, 154) and the rodent yolk sac (119).

Decreased renal megalin expression has been demonstrated in some diseases characterized by proteinuria. These include Dent’s disease, which is caused by mutations in the renal CI channel CIC-5. Disruption of CIC-5 impairs proximal tubule endocytosis and causes a reduction in megalin expression in some knockout mouse models and in some reports of human cases (29, 105, 108), although the findings in humans remain to be confirmed.

Much of our knowledge on the functions of megalin is based on data recovered from the study of megalin-deficient mice. These mice, produced by gene targeting, exhibit severe forebrain abnormalities as well as lung defects (150). Most of them die perinatally; however, some survive to adulthood, constitut-
ing a model for the study of megalin function. Additional evidence has been recovered from mice with targeted kidney-specific knockout of the megalin gene (77). Megalin-deficient proximal tubule cells are characterized by a loss of endocytic invaginations, vesicles, and the membrane-recycling compartment, dense apical tubules (DAT) (31). So far, no significant changes in transport of water, electrolytes, glucose, or amino acids have been described in megalin-deficient mice; however, an increased amount of a number of low-molecular-weight serum proteins has been identified in urine. Analyses of megalin knockout mice and patients with low-molecular-weight tubular proteinuria, including Dent’s disease, have shown several analogies (78, 105).

Little is known about the regulation of megalin gene expression in vivo; however, receptor-associated protein (RAP), a 45-kDa protein modulating posttranslational protein processing (22, 23, 149, 152) and shown to bind megalin (30) as well as other members of the LDL-receptor family, is required for normal expression and subcellular distribution of megalin in kidney proximal tubules (12). RAP inhibits binding of almost all ligands to megalin, constituting an important tool for the study of megalin function. A HNEL endoplasmic reticulum (ER) retention signal has been identified in RAP (22) targeting it mainly to the ER, where it recycles between the ER and the cis-Golgi (22). RAP serves as a chaperone protecting newly synthesized LRP from the early binding of ligands, which are also synthesized by the cells (Fig. 5) (22, 23, 149, 152). Such premature binding of ligands within the ER may cause receptor aggregation and retention. In addition, RAP may be involved in folding of the receptors (Fig. 5) (23). RAP deficiency is associated with a reduction in megalin expression to ~25% of normal and a change in subcellular distribution, causing a reduction in brush-border expression and an accumulation of megalin in intracellular compartments including the ER and the paramembranous ER (12). This strongly suggests that RAP serves a similar chaperone function for megalin. RAP-deficient mice are viable and fertile with no overt abnormalities in renal function (149). In contrast to megalin-deficient proximal tubule cells, no changes in the ultrastructural appearance of the endocytic apparatus were observed in RAP-deficient mouse kidneys (12). Detailed analysis including two-dimensional gel electrophoresis showed increased urinary excretion of specific proteins, including DBP and α-amylase (12). Thus RAP-deficient mice, having a ~75% reduction in megalin expression, exhibit low-molecular-weight proteinuria and have been used to study megalin function in vivo.

Cubilin. Cubilin is a multiligand endocytic receptor. The first ligand for cubilin was not identified until 1997, when it was recognized as the intestinal IF-B12 receptor (126). Since then, a number of other ligands have been described (Table 1), of which many may be filtered in the renal glomerulus and reabsorbed by cubilin-mediated endocytosis, including albu-
min, immunoglobulin light chains, apolipoprotein A-I, transferrin, DBP, hemoglobin, and myoglobin.

STRUCTURE. Cubilin was originally identified as the target of teratogenic antibodies in rats (Fig. 2). It is a 460-kDa protein with little structural homology to other known endocytic receptors (Fig. 3) (72, 92). Cubilin has no transmembrane domain, and it can be released from renal cortical membranes by nonenzymatic and nonsolubilizing procedures (92). It is composed of a NH₂-terminal 110-amino acid region necessary for membrane anchoring of the receptor (74) followed by eight EGF-like repeats and 27 complement subcomponents C1r/C1s, Uegf, and bone morphogenic protein-1 (CUB) domains. The structure of the CUB domains has been studied in spermadhesin. They are organized as barrel-like structures containing two, five-stranded β-sheets connected by surface-exposed β-turns (115), which may be arranged so that the less-conserved surface of the β-turns is externally exposed for ligand binding. The many CUB domains in cubilin support the ability of the receptor to bind a variety of ligands. Binding sites for selected ligands have been partially localized. A binding site for IF-B₁₂ has been located within CUB domains 5–8, whereas the binding site for RAP has been located within CUB domains 13–14 (74). Other studies have suggested additional IF-B₁₂ as well as albumin binding sites in a 113-residue NH₂ terminus. Although IF-B₁₂ inhibits binding of albumin to cubilin, the binding sites for the two proteins do not appear to be identical within this domain (155). Finally, megalin appears to bind to the NH₂-terminal region including CUB domains 1 and

![Fig. 4](image-url) Electron micrograph showing the distribution of megalin, FR, and cubilin in the apical parts of a cryosectioned, rat kidney proximal tubule cell. Megalin, FR, and cubilin are identified by triple-labeling immunocytochemistry using polyclonal sheep anti-megalin (1:25,000), polyclonal rabbit anti-FR (1:2,000), and monoclonal mouse anti-cubilin (1:5,000) followed by 3 different gold-conjugated secondary antibodies (1:50): donkey anti-sheep (10 nm), donkey anti-rabbit (18 nm), and donkey anti-mouse (6 nm). Megalin (filled arrowheads), FR (open arrowheads), and cubilin (arrows) colocalize in the microvilli (MV), endocytic invaginations (INV), endocytic vesicles (EV), and dense apical tubules (DAT) constituting the membrane-recycling compartment in the kidney proximal tubule. Insets: selected structures, marked by rectangles, enlarged for better identification of gold particles. The rat kidney was fixed by perfusion with 4% paraformaldehyde, infiltrated with sucrose, and frozen before cryosectioning and immunolabeling. Bar = 0.2 μm.

![Fig. 5](image-url) Role of RAP in the processing of megalin. RAP binds to the newly synthesized receptor in the endoplasmic reticulum (ER), thus preventing early binding of ligands also present in the ER. As megalin is transported through the Golgi, RAP dissociates and is retrieved to the ER by vesicular transport following binding to specific receptors. It is suggested that ligand binding to megalin in the Golgi and later transport vesicles is prevented by a lower pH in these compartments. Furthermore, the binding of RAP may assist formation of intradomain disulfide bounds and proper folding of the receptor in the ER, as suggested for LDL receptor-related protein (LRP). From Ref. 27 with permission from Nature Reviews Molecular Cell Biology (2002), Macmillan Magazines, as modified from Refs. 23 and 148.
amounts of proteins (up to 140 kDa) reactive with anti-cubilin overall expression to the changes in megalin expression, showing decreased cubilin was reduced in RAP-deficient mice, somewhat similar observations in our laboratory showed that the expression of cubilin seems more restricted than megalin, the two receptors are coexpressed in a number of epithelia, including the ileum and the rodent yolk sac (27).

Normal expression of cubilin is dependent on AMN, a ~45-kDa transmembrane protein (Fig. 3) identified as an important factor for the normal development of the middle portion of the primitive streak in mice (65). Previously, a 40- to 45-kDa protein of unknown nature was shown to coeluete with cubilin when purified from kidney by IF-affinity chromatography (11). Later studies suggested this protein to be AMN (40). Cubilin and AMN colocalize in kidney proximal tubule. Cotransfection of cubilin fragments and AMN into Chinese hamster ovarian (CHO) and Madin-Darby canine kidney cells shows that AMN interacts with the EGF-type repeats of cubilin and is essential for normal translocation of the cubilin-AMN complex from the ER to the plasma membrane and for the subsequent endocytosis (34, 40). Furthermore, dogs with a mutation in the AMN gene (52, 53) as well as AMN-deficient mouse epithelial cells reveal defective apical insertion of cubilin (6, 41, 136). Mutations in either the cubilin or the AMN gene have been identified in Imersenlund-Gräbeck disease (2, 138), a rare inherited vitamin B_{12} deficiency syndrome characterized by defective intestinal absorption of IF-B_{12} (21, 144) and an apparent geographical concentration in Scandinavia and the Middle East (139). Cubilin gene mutations were identified in Finnish and Arab families (2, 139). In two Finnish families, this involved either a one-amino acid substitution in CUB domain 8 affecting the binding of IF-B_{12} or a point mutation expected to activate a cryptic intronic splice site causing an in-frame insertion with several stop codons, predicting a truncation of the receptor in CUB domain 6 (2, 73). Whereas the patient with the latter mutation has overt proteinuria, patients with amino acid substitution reveal varying degrees of proteinuria ranging from little or no, to clear-cut (144). Affected members of three Norwegian families, shown to have a nucleotide deletion causing the introduction of an early stop codon in the AMN gene (138), were all previously characterized and had proteinuria at the time of diagnosis. Other mutations have been identified in the AMN gene; however, the renal phenotype of these patients is not clear (139). Based on the variety of mutations shown to cause Imersenlund-Gräbeck syndrome, it may by hypothesized that the difference in renal phenotype, in particular the degree and type of proteinuria, reflects the degree of inactivation of cubilin function, in particular whether the mutation affects the multiligand properties or only the IF-B_{12}-binding site.

Like megalin, cubilin binds RAP (11); however, the function of RAP binding to cubilin remains unclear. Unpublished observations in our laboratory showed that the expression of cubilin was reduced in RAP-deficient mice, somewhat similar to the changes in megalin expression, showing decreased overall expression to ~25% of controls. In addition, increased amounts of proteins (up to 140 kDa) reactive with anti-cubilin antibodies were observed in the urine of RAP-deficient mice. Whether this was due to a direct role of RAP in the expression of cubilin, or reflects the reduced expression of megalin, remains to be established.

**FRs.** FRs are membrane-anchored proteins binding folate with high affinity. They were originally identified as a soluble FBP in milk (Fig. 2) (44), and later their presence was established in both serum and tissues (4, 54, 67). The term folate receptor has been introduced to indicate its function in cellular folate uptake (4).

**Structure.** FRs are glycosylated ~40-kDa proteins binding folate with high affinity (K_{d} ~1 nM) (4, 54, 67). At least four FR isoforms have been identified and characterized in humans. FRα and FRβ are membrane-associated, GPI-linked proteins (Fig. 3) (75, 141) expressing different affinities for different stereospecific folate analogs (145). They may be enzymatically released from the membranes (38, 75, 82). FRγ is specific for hematopoietic cells and present in serum (130). The fourth human FR gene (FRδ) predicts a 27.7-kDa protein with a unique expression pattern in both adult and embryonic tissues (133). Whereas at neutral pH FRs bind most naturally occurring folates, dissociation is rapid at low pH. Binding of folate to the FR induces conformational changes, increasing stability and decreasing hydrophobicity of milk FBP (64).

**Renal expression.** FRs are heavily expressed in kidney proximal tubule brush-border membranes (Fig. 4) (9, 58, 59, 66, 129) and have been identified in the mouse glomerulus (14) and in human urine (50). Immunocytochemical studies have localized the FR to the proximal tubule brush border, endocytic invaginations, including coated pits, endocytic vesicles, and DAT (9, 58). Identification of mRNA suggests that the adult human kidney expresses both FRα and FRβ (116). In addition to the kidney, FRα is present in other, predominantly epithelial, cells, whereas FRβ is expressed at low to moderate levels in several different tissues (4, 54, 75, 116).

Targeted gene knockout of folbp1 and folbp2 (the mouse equivalents to FRα and FRβ) has confirmed a role of the FR in folate metabolism (107) and renal folate transport (10). Deletion of folbp1 is lethal and associated with changes in serum folate and embryonic defects that can be rescued by supplementing the dams with folate (107, 134). The expression of FRs is regulated by extracellular folate concentration. In vitro studies suggest that FRs are upregulated when cells are grown under low-folate conditions (55, 68), whereas in the kidney FRs are downregulated in mice and rats fed a low-folate diet (35, 43).

**Interaction among megalin, cubilin, and FBP.** A metabolic interaction between folate and B_{12} is well established. Recent studies have indicated additional interaction between proteins involved in the uptake of these vitamins, showing binding of both cubilin and FBP to megalin. A high-affinity, Ca^{2+}-dependent, and partially RAP-inhibitable binding between megalin and cubilin has been demonstrated in vitro (92). Megalin deficiency is associated with reduced brush-border expression of cubilin (6), and in megalin-deficient mice the cubilin ligand transferrin is accumulated at the proximal tubule luminal membranes, revealing defective uptake (71). Also, the uptake of another cubilin ligand, HDL, is inhibited in vitro by anti-megalin antibodies as well as by megalin anti-sense oligonucleotides (49). This indicates that megalin may mediate the cointernalization and possibly recycling of cubilin. In addition, megalin and cubilin share a number of ligands,
involved in intracellular translocation of FRs, possibly regulating luminal expression of membrane FR by internalization and degradation.

**Proximal Tubule Uptake of Folate and Vitamin B₁₂**

Proximal tubule uptakes of both folate and vitamin B₁₂ were recognized early and shown to be saturable processes (46). At physiological serum concentrations, the estimated amounts of vitamins filtered in the glomeruli exceed the recommended daily intake, whereas the urinary excretion of both intact folate and vitamin B₁₂ is minimal. Thus proximal tubule receptor-mediated uptake efficiently prevents urinary losses. Recent studies have provided significant information on the molecular mechanisms responsible for this, involving the endocytic receptors presented above.

**Vitamin B₁₂.** Megalin is essential for the proximal tubule reabsorption of filtered TC-B₁₂ (13), mediating endocytosis of TC-B₁₂ (90, 104). Megalin and TC colocalize within the endocytic apparatus of rabbit kidney proximal tubule cells (142). TC-B₁₂ binds to megalin with an estimated affinity (Kₐ) of ~183 nM when analyzed by surface plasmon resonance (SPR) analysis, also showing a possible second binding site (Kₐ ~1.4 μM). Binding to immobilized megalin in microtiter trays revealed a half-maximum binding of 12.5 nM, indicating that the affinity may be higher than estimated by SPR analysis. The importance of megalin for the tubular reabsorption and renal accumulation of TC-B₁₂ was established using megalin knockout mice (13). These revealed increased urinary excretion of B₁₂ and a 28-fold increase in renal B₁₂ clearance, along with a 4-fold decrease in the B₁₂ content of megalin-deficient mice kidneys. Immunocytochemistry in wild-type mice showed that most of the reabsorbed vitamin was located in the very early part of the proximal tubule, indicating efficient reabsorption (13).

An additional 62-kDa TC-B₁₂ receptor has been identified in the kidney and other tissues, including placenta, liver, and intestine (16–18). This receptor is a glycosylated 45-kDa single polypeptide with a yet unknown primary structure. It is normally present as a 124-kDa dimer in both apical and basolateral membranes of kidney proximal tubule, however, with a 90% distribution to the basolateral membranes when estimated by membrane fractionation (17, 18). The expression appears to be regulated by corticoids (17). Its role in TC-B₁₂ uptake is supported by the low tissue B₁₂ and apparent vitamin B₁₂ deficiency developing in rabbits following injection with an antiserum against this receptor (16) and by the decrease in renal uptake of orally administered labeled B₁₂ in adrenalec-tomized rats (17). It was recently suggested that this ubiquitous TC-B₁₂ receptor binds to megalin and that this binding is associated with increased binding of TC-B₁₂ to the purified receptors (153). It was also shown that the immunization of rabbits with megalin was associated with decreased expression of the TC-B₁₂ receptor in purified apical kidney membranes, leading to the suggestion that megalin is involved in the apical targeting of this receptor. This proposed functional and structural interaction between megalin and the 62-kDa TC-B₁₂ receptor awaits further clarification. Evidence suggests that the major mechanism for renal accumulation of vitamin B₁₂ is by tubular reabsorption of filtered TC-B₁₂. Thus the 10–90% distribution of the 62-kDa TC-B₁₂ receptor in apical vs. basolateral renal membranes is puzzling; however, it may suggest an alternative role for the 62-kDa receptor in the kidney, e.g., in basolateral uptake or secretion. The distribution of labeled B₁₂ injected during vitamin depletion as well as during vitamin load fits a model of cellular TC-B₁₂ uptake involving two distinct receptors: a possibly regulated ubiquitous TC-B₁₂ receptor mediating saturable cellular uptake, and a luminal tubule receptor mediating constitutive high-capacity reabsorption of filtered TC-B₁₂ (7, 90).

Although both megalin and cubilin mediate endocytic uptake of vitamin B₁₂, and both are heavily expressed in proximal tubule epithelial cells, a role for cubilin in kidney B₁₂ uptake has not been established. Cubilin purified from kidney binds IF-bound B₁₂, and uptake of IF-B₁₂ in the kidney is inhibited by anti-cubilin antibodies (11). Minute amounts of IF can be detected in human serum and may be filtered, as IF has been identified in urine (111, 143). However, due to the very small amounts of B₁₂ filtered in complex with IF, the significance of cubilin for renal uptake of B₁₂ is dubious.

**Folate.** A role of the FR in renal tubular folate uptake was hypothesized when FRs were identified in the kidney proximal tubule (66, 129) and further supported by kinetic studies showing that the urinary clearance of folate derivatives was inversely related to their affinity for the FR (127). The importance of the FR was established by the analysis of renal folate handling in mice with targeted gene knockout of folbp1 and folbp2 (10). Mice defective in folbp1 (equivalent to human FRα) reveal a significant increase in renal folate clearance at both low-folate and normal-folate intakes, showing impaired tubular uptake of filtered folate (10). It was calculated that the amount of folate excreted in the urine of folbp1 null mice is ~100 times higher than in wild-type mice, indicating that the tubular reabsorptive capacity related specifically to folbp1 in the low-folate situation is ~4 nmol/24 h in mice. With an estimated 20,000 nephrons in the mouse, this corresponds to a transport rate of ~0.15 fmol·min⁻¹·nephron⁻¹. Folbp2 (equivalent to human FRβ) deficient mice revealed lower serum folate levels compared with wild-type but no significant changes in urinary folate clearance (10). Thus, although mRNA corresponding to both the human equivalents of folbp1 and folbp2 has been demonstrated in the human kidney (116), the role of folbp2 in renal folate reabsorption remains unclear.

The rate of transstubular folate transport has been estimated using several other approaches. Perfusion of isolated rabbit proximal tubules revealed a reabsorptive capacity of 4.0 fmol·min⁻¹·nephron⁻¹, which should be compared with an
estimated rabbit single-nephron filtration of folate ~3 fmol/min (8). Micropuncture of single rat proximal tubules for 1 min with $[^{1}H]$_folic acid demonstrated tubular uptake at a rate of 0.65 fmol/mm tubule or 4.5 fmol/proximal tubule (128), close to the observed rate in isolated perfused rabbit tubules (8). In contrast, in vitro studies using cultured human proximal tubule cells suggested a specific apical-to-basolateral folate transport rate of only 0.12 fmol·min$^{-1}$·cm$^{-2}$ (89), considerably lower than that observed with perfused rabbit proximal tubules, indicating either lower activity of cells in vitro, possible effects of flow rate in micropertusion studies, or species differences, conceivably reflecting differences in serum folate levels. Studies using cultured human proximal tubule cells have suggested bidirectional transport (95), but tubular secretion of folate in vivo has not been established.

Uptake of folate by the FR is suggested to involve internalization of the FR-folate complex (129). Studies in monkey kidney MA104 cells and other cell lines indicated that binding is followed by invagination of the FR into caveolae rather than the clathrin-coated pit pathway (3). As the folate-FR complex clusters into caveolae, this is followed by acidification (76) and dissociation of the ligand (3). The FR is recycled to the plasma membrane by reopening of the caveolae. Later in vitro studies have challenged this hypothesis by showing that GPI-linked receptors concentrate in caveolae by cross-linking with antibodies, but not with folate (87, 106), that FRs can be endocytosed following stimulation (106), and that in KB cells derived from a carcinoma of the human nasopharynx most internalized FR bypasses caveolae (114). Experiments in CHO cells have suggested that FRs are internalized into a distinct GPI-anchored, protein-enriched compartment independent of clathrin (118). Thus FRs may be internalized by several different endocytic pathways depending on cell type and on the stimulus evoking internalization. Micropuncture of kidney proximal tubules showed endocytosis of folate-gold particles into coated pits and vesicles (8), and the FR has been localized to the compartments of the classic endocytic and recycling pathway (9, 14, 58). Also, caveolae are almost never observed in kidney proximal tubule cells (20), making it unclear whether this mechanism operates in these cells. Micropuncture with anti-FR antibodies (9), as well as kinetic studies (128), suggests rapid recycling of FRs to the plasma membrane for regeneration of folate-binding sites.

Several other mechanisms have been implicated in renal tubular folate transport, including dual-component transport systems (96) or even nonspecific pathways (97). Folate uptake has been studied in vitro using a number of different cell systems (4, 54, 67). In certain cell types, including KB cells and MA104 cells, folate uptake apparently depends on FRs. Other cell lines, i.e., mouse L1210 leukemia cells, utilize a high-capacity folate uptake system mediated by the reduced folate carrier (RFC). Thus folate uptake may be mediated by a receptor-mediated mechanism depending on the FR and/or carrier-mediated mechanisms, of which the RFC has been most extensively characterized (86). The RFC has been located to basolateral membranes in kidney tubules (146), suggesting that the RFC may be involved in basolateral folate uptake or in the cellular exit of reabsorbed folate. RFC represents a high-capacity, low-affinity transport system for folate with a $K_I$ for 5-MTHF on the order of 2 μM. Targeted gene knockout of RFC is embryonically lethal; however, a limited number of mice can be rescued by folate supplementation of the dams. These mice revealed defects in erythro- and lymphopoiesis along with abnormalities in renal and seminiferous tubule development (157). Additional organic anion transporters, which may also transport folate, have been identified in the kidney (86). Thus a carrier-mediated mechanism may be responsible for the transport of folate out of endosomes, as well as for the exit of folate from the tubule cells.

In addition to the FR, megalin may also be involved in the tubular uptake of folate. SPR analysis, autoradiography, and uptake studies suggested that megalin mediates binding and internalization of soluble FBP (14). Folate bound to serum FBP is filtered as a complex. It may be estimated that <1% of filtered FBP is excreted (14), suggesting efficient tubular reabsorption by megalin-mediated endocytosis similar to other vitamin carrier proteins (27, 31). Approximately 108 nmol or ~48 μg of FBP-bound folate may be recovered daily by megalin-mediated uptake, which could be important in individuals with low-folate intake. Thus FBP is involved in folate uptake both as a GPI-linked, membrane-associated receptor for filtered free folate and as a filtered, soluble, folate carrier protein binding to megalin.

A potential role for cubilin in the recovery of filtered folate is suggested by the important role of this receptor for the reabsorption of filtered albumin (6). Albumin binds to cubilin with an estimated $K_d$ of ~0.6 μm (6). Because albumin is a carrier of folate, along with other vitamins, cubilin may be involved in the uptake of folate, depending on the amount of filtered albumin. The significance of this remains to be established both in the normal kidney and under pathological conditions characterized by increased filtration of albumin.

**Postendocytic Processing in the Kidney**

Following internalization into renal proximal tubule cells, vitamins may be metabolized, stored, or released (Fig. 6). A recent study has shown endocytosis and lysosomal accumulation of a fluorescent folate probe using in vivo two-photon microscopy (121). Following intravenous injection of labeled folate into rats, there is a transient increase in the accumulation of label within the kidney at levels exceeding the liver; however, 24 h after injection, the amount of labeled folate within the kidney is significantly reduced by redistribution into other tissues (131), indicating that the kidney does not accumulate large amounts of folate. The endocytosed free folate is transported to the cytoplasm, possibly by a carrier protein (109). The reabsorbed folate may, in part, undergo metabolic transformation into other folate forms but is catabolized only to a very small extent (88, 96). Alternatively, folate may be transported across the tubular cells in a vesicular compartment, as suggested based on the finding of fluorescent-labeled folate in basolateral vesicles of the proximal tubule (121). However, no morphological evidence of such transport was observed following proximal tubule micropuncture with folate-gold particles (8) or radiolabeled folic acid (58).

Substantial amounts of B12 accumulate in the rodent kidney, especially in states of vitamin load (7, 13, 47, 51, 124). In vitro TC is degraded within 24 h, whereas B12 accumulates and is only slowly released (79, 104). Studies on the distribution of a single oral or parenteral dose of labeled B12 suggest that high
doses of the vitamin are retained in the kidneys weeks after injection (51). Although traditionally the liver is considered the major storage organ for B12, it has been shown that the ratio of injected labeled B12 to total B12, estimated as microbiologically active B12, is higher in the kidney than in other organs up to 17 days after injection, suggesting that the injected B12 is retained in the kidney for that long (47). Internalized vitamin B12 accumulates in rodent proximal tubule lysosomes, predominantly as free vitamin (7, 13, 100), suggesting that this organelle may serve a storage function. When injection of a single dose of radiolabeled B12 was followed by injections of large doses of B12, more vitamin was retained in the kidneys up to 30 days after injection (51). This may indicate that the release from the kidneys is negatively regulated by vitamin status, but it may also reflect reabsorption of increased amounts of filtered, labeled B12 by the constitutive high-capacity, megalin-mediated mechanism. Much less free vitamin is identified in vitamin-depleted animals (7, 13), indicating that the transport out of lysosomes may constitute a rate-limiting, and perhaps regulated, step in the transtubular vitamin transport. A pH-sensitive, lysosomal membrane-associated B12 transporter has been identified in liver, suggesting that B12 may be transported out of the lysosomes for further transport and/or processing in the cytosol (62). This is supported by the identification of an inborn error of cobalamin metabolism causing the lysosomal accumulation of B12 (140), possibly due to a defective lysosomal membrane transporter. Studies have suggested that injected and reabsorbed cyanocobalamin is metabolized within the kidney (103, 104), possibly in the cytosol or the mitochondria following transport out of lysosomes.

Alternatively, B12 may be transported along with TC across the tubular cell. Megalin-mediated transcellular transport of intact protein has been suggested for thyroglobulin (85) and for RBP (84). So far, this has not been confirmed in vivo, and the accumulation of free vitamin B12 along with rapid degradation of internalized TC in cultured proximal tubule cells strongly suggest that only the vitamin is transported through the cells. The mechanism, by which reabsorbed B12 is released from the tubular cells following reabsorption, is largely unknown. Both TC and HC, and possibly IF, are synthesized in proximal tubule cells in vitro (19, 104, 112). TC mRNA was identified in adult porcine and human kidney, whereas HC mRNA could not be identified in the adult porcine kidney (104). Whether B12 and carrier proteins are combined within the cell before secretion or are secreted separately remains to be established.

Fig. 6. Schematic representation of a kidney proximal tubule cell showing possible pathways by which megalin, cubilin, and FR are involved in endocytosis and transcellular transport of folate and vitamin B12. Filtered vitamins, free or complexed to carrier proteins, bind to megalin and/or cubilin, or FR, followed by endocytosis. AMN and/or megalin facilitates the endocytosis of cubilin. The ligands are released from the receptors by the low pH in the endosomes, and receptors recycle through a membrane-recycling compartment (DAT). The protein component of carrier molecules is degraded in lysosomes, whereas the vitamin is transported across the epithelial cell. A minor fraction of receptors may be transported to lysosomes for degradation. Endo- or lysosomal membrane transporters for cobalamin and folate have been suggested. FR-mediated transcellular transport (right) has been suggested in the kidney. Pathways or interactions yet to be established are indicated by ?. 

Invited Review
THE KIDNEY IN B12 AND FOLATE HOMEOSTASIS

AJP-Renal Physiol • VOL 291 • JULY 2006 • www.ajprenal.org

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CONCLUSIONS AND FUTURE PERSPECTIVES

By mediating endocytosis of filtered vitamins and specific carrier proteins, both megalin and the FR play an important role in the conservation of vitamin B12 and folate. In addition, cubilin may be involved by mediating uptake of albumin, an additional carrier of folate. Functional interaction between receptors and binding proteins expands the already established metabolic relation between the vitamins. The unraveling of the molecular basis for renal folate and B12 uptake has shown this to be a constitutive process, similar to the uptake of other vitamin carrier proteins, causing vitamins to locate in specific vesicular compartments. Significant amounts of free B12 appear to accumulate in kidney lysosomes, suggesting that this serves a storage function, and, in addition, vitamins are metabolized within the kidney, suggesting a role in vitamin homeostasis.

The mechanism by which vitamins are further transported, processed, and released by the proximal tubule cells is largely unresolved (Fig. 6), although it may involve specific carriers associated with intracellular vesicular and basolateral membranes, as well as proximal tubule synthesis and secretion of carrier proteins. Further studies, including the use of inherited and genetically engineered models of defective vitamin transport as well as transfection studies with fluorescent-labeled carrier proteins, should provide clues to resolve these mechanisms and their possible regulation. The cytoplasmic tail of megalin contains potential signaling motifs as well as domains interacting with cytosolic proteins (27, 42, 80, 94, 98, 158). Future studies may reveal signaling pathways, as has been implicated for other members of the LDLR family (56), possibly also involved in regulation of vitamin transport.

Most of the evidence establishing the mechanisms for renal tubular uptake of folate and B12 is based on experiments in animals, particularly in rodents. Studies in humans, including patients with renal disease and proteinuria as well as patients with inherited, specific receptor defects, should assess the importance of these mechanisms to vitamin homeostasis in humans.

ACKNOWLEDGMENTS

The author thanks Professor Erik I. Christensen, Professor Søren K. Moestrup, and Professor Ebba Nexø for constructive and inspiring comments regarding the manuscript.

GRANTS

This work was supported by the Danish Medical Research Council, the University of Aarhus, the NOVO-Nordisk Foundation, Fonden til Lægevidenskabelgens Fremskred, the Biomembrane Research Center, the Beckett Foundation, the Leo Nielsen Foundation, the Ruth König-Petersen Foundation, and the Birn Foundation.

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