The kidney in vitamin B₁₂ and folate homeostasis: characterization of receptors for tubular uptake of vitamins and carrier proteins

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Birn, Henrik. The kidney in vitamin B₁₂ 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 B₁₂, and their carrier proteins. The multiligand receptor megalin is important for the uptake and tubular accumulation of vitamin B₁₂. During vitamin load, the kidney accumulates large amounts of free vitamin B₁₂, suggesting a possible storage function. In addition, vitamin B₁₂ 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-B₁₂ 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.

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

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 B₁₂ 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 B₁₂ 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.

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 p-aminobenzoic acid. Additional glutamate residues are attached to the 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 is used for the synthetic pteroylglutamic acid representing fully oxidized folate. The biologically active form is reduced tetrahydrofolate (THF)¹, serving as an essential cofactor in methylation reactions, including the vitamin B₁₂-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.

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.
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 glutamylcarboxypeptidase and absorbed as such. Various folate derivatives, i.e., 5,10-methylene-THF, 10-formyl-THF, and 5-MTHF, serve as 1-carbon donors in biochemical reactions involving the synthesis of purines, thymidine, and the conversion of homocysteine to methionine. The interconversion between various folate forms involves intermediate forms and reactions not shown. The major source of 1-carbon units for folate metabolism in mammalian cells is the conversion of serine to glycine. Serum B\(_{12}\) is normally coupled to the binding proteins transcobalamin (TC) and haptocorrin (HC); however, most cells take up only TC-B\(_{12}\). Following internalization, B\(_{12}\) may be metabolized into the biological active forms methylcobalamin and 5-deoxyadenosylcobalamin by reactions involving reduction of the cobalt atom (indicated by \(\text{Co}^{2+}\) and \(\text{Co}^{1+}\)) and various enzymes not shown. 5-Deoxyadenosylcobalamin serves as a cofactor for the mitochondrial methylmalonyl-CoA-mutase catalyzing the conversion of methylmalonyl-CoA into succinyl-CoA, important for the oxidation of odd-chain fatty acids and for the degradation of certain amino acids. Methylcobalamin, initially formed by methylation of enzyme-bound B\(_{12}\), 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 B\(_{12}\) (blue arrows). The transfer of a methyl to homocysteine causes reduction of methionine synthase-bound B\(_{12}\) 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 B\(_{12}\) deficiency may lead to elevated homocysteine due to inhibited conversion of this into methionine. Vitamin B\(_{12}\) deficiency may also lead to elevated methylenomononic acid (MMA) as a result of accumulation of methylmalonyl-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 B\(_{12}\) deficiency. This results in a functional folate deficiency despite elevated 5-MTHF levels. Thus both folate and vitamin B\(_{12}\) deficiency may lead to inhibited synthesis of nucleotides for RNA and DNA, causing megaloblastic anemia. Vitamin B\(_{12}\) 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-B\(_{12}\) 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.
folate bound to high-affinity serum proteins varies between species, being high in pigs (83) and relatively low in rodents such as mice and rats (127). The concentration of FBP in human serum is estimated to be 0.6 nM (61) compared with a normal serum folate concentration of 5–20 nM. The affinity of FBP for folate in serum is ~0.1 nM, suggesting that serum FBP is fully saturated. The amount of folate associated with serum proteins has been estimated to be ~20% in rats within a wide serum folate concentration range (127). In humans, the fraction of protein-bound folate has been estimated to be from ~20% (156) to ~65%, being constant within a wide concentration range.

**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 cobalamin derivative. Vitamin B12 serves as a cofactor in two essential biochemical reactions involving 1) the formation of methionine from homocysteine bycytoplasmic methionine synthase using 5-MTHF as one carbon donor, and 2) the interconversion of methylmalonyl-CoA to succinyl-CoA by mitochondrial methylmalonyl-CoA mutase (Fig. 1). Thus B12 deficiency is associated with accumulation of homocysteine, methylenic acid (MMA), and, in some cases, also 5-MTHF as a result of the trapping of 5-MTHF due to decreased methionine synthase activity. Clinically, B12 deficiency results in megaloblastic anemia, indistinguishable from that caused by folate deficiency, and neurological dysfunction. The total body content of vitamin B12 varies; however, most estimates are ~2–3 mg, with 1–1.5 mg in the liver, classically considered the major organ for B12 accumulation (1, 45). Mammals are unable to synthesize vitamin B12 and thus rely on dietary intake; however, they are able to convert the different forms of vitamin B12 into the active forms, methylcobalamin and 5-deoxyadenosylcobalamin. Vitamin B12 is synthesized by microorganisms, particularly in the gastrointestinal tract of herbivorous animals, and absorbed. Thus the major food sources of the vitamin are liver, meat, fish, dairy products, eggs, and shellfish.

After ingestion, vitamin B12 is released from dietary protein by the acidic environment and peptic digestion in the stomach, followed by binding to haptocorrin (HC), a ~65-kDa glycoprotein secreted in saliva and gastric juice and favored by the acidic environment. In the duodenum, pancreatic secretion raises intraluminal pH and facilitates degradation of HC by pancreatic proteases, causing vitamin B12 to bind to intrinsic factor (IF; molecular mass ~50 kDa) secreted by the parietal cells of the gastric mucosa and by the pancreas (125). The IF-B12 complex is absorbed in the ileum by binding to the intestinal IF-B12 receptor, the cubulin-amnionless (AMN) complex (40, 126). The binding to the receptor depends on B12 binding to IF (11). Following absorption, B12 is released from IF within the enterocyte (113). In serum, B12 is bound to transcobalamine (TC), a 45-kDa nonglycosylated serum protein, and HC. Normal B12 concentration in human serum is estimated to be ~0.3 nM (102) compared with ~1.1 nM in normally fed laboratory rats (7). The concentration of TC in normal human serum is ~1 nM, of which ~10% is saturated, depending on vitamin intake (15), whereas the concentration of HC is ~0.4 nM (93), of which ~75% is saturated (101). Thus normally no free B12 is present in the circulation. Although TC binds only ~25% of the circulating vitamin, it is responsible for the delivery of B12 to most tissues. Indeed, whereas inherited TC deficiency is associated with severe megaloblastic anemia, patients with HC deficiency may be asymptomatic with normal levels of MMA despite low total serum B12 (110). This is also reflected in the half-life of the two carrier proteins, being <2 h for TC compared with several days for HC. HC binds to the asialoglycoprotein receptor, and in adults it is probably only taken up in significant amounts in the liver (24). 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 insulin (46), suggesting a filtered load of free folate of ~1 mg/24 h in humans (127). A
small fraction of folate is bound to serum FBP. The molecular mass of ~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 B₁₂ similarly is dependent on serum protein binding and thus on the concentration of B₁₂ in serum. In humans, no urinary excretion of B₁₂ was detected at concentrations <1.1 ng/ml (~800 pM), whereas at concentrations >12 ng/ml (~8 nM) B₁₂ is excreted at a rate similar to the glomerular filtration rate (147). Thus unbound B₁₂ is freely filtered and may in fact serve as a marker to estimate glomerular filtration rate (99). At normal serum B₁₂ concentration, the filtered load of the vitamin in humans has been estimated to be 1.5 μg (79). The molecular mass of TC suggests that the TC-B₁₂ complex is filtered, supported by the demonstration of small amounts of B₁₂-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 B₁₂, 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 B₁₂ and folate.

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

<table>
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<tr>
<th>Vitamin carriers</th>
<th>Cubilin</th>
<th>Megalin</th>
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<tr>
<td>Transcobalamin-vitamin B₁₂</td>
<td>Intrinsic factor-vitamin B₁₂</td>
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<td>Vitamin D-binding protein</td>
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<td>Retinol-binding protein</td>
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### Other carrier proteins

| Albumin | Albumin |
| Myoglobin | Myoglobin |
| Hemoglobin | Hemoglobin |
| Lactoferin | Transferrin |
| Neutrophil-gelatinase-associated lipocalin | Oderant-binding protein |
| Transthyretin | Liver-type fatty acid-binding protein |
| Sex hormone-binding globulin | |

### Lipoproteins

- Apolipoprotein B
- Apolipoprotein Al
- Apolipoprotein A
- Apolipoprotein E
- Apolipoprotein J
- Apolipoprotein H
- Apolipoprotein M

### Enzymes and enzyme inhibitors

- Plasminogen activator inhibitor-type 1
- Plasminogen activator inhibitor-type 1
- 1-urokinase
- Plasminogen activator inhibitor-type 1
- 1-tissue plasminogen activator
- Pro-urokinase
- Lipoprotein lipase
- Plasminogen
- α1-Amylase
- α1-Microglobulin
- Lysozyme

### Immune- and stress-response related proteins

- Immunoglobulin light chains
- Immunoglobulin light chains
- Pancreatitis-associated protein 1
- Clara cell secretory protein
- Advanced glycation end products
- β₂-Microglobulin

### Receptors and transmembrane proteins

- Cubilin
- Megalin
- AMN
- Heavy metallothionein
- Cation-independent mannose-6-phosphate receptor

### Drugs and toxins

- Aminoglycosides
- Polymyxin B
- Aprotinin
- Trichosantin

### Others

- Cytochrome c
- Cytochrome c
- Receptor for seminal vesicle secretory protein II

**AMN, amnionless.**
ligands (Table 1). Ligands for megalin that may be filtered in
the glomeruli include vitamin D-binding protein (DBP), reti-
nol-binding protein (RBP), TC-B12, FBP, parathyroid hor-
mone, insulin, epidermal growth factor (EGF), prolatin, albu-
mmin, hemoglobin, myoglobin, β2- and α1-microglobulin, apo-
lipoprotein H, lysozyme, cytochrome c, and α-amylase.

STRUCTURE. Megalin was originally identified as the antigen
in Heymann nephritis, a rat model of membranous glomerulo-
nephritis (Fig. 2) (69). It is a 600-kDa protein (Fig. 3) with a
large NH2-terminal extracellular domain, a single transmem-
brane 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 kid-
ney 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 identi-
fied 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 Cl
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 fore-

brain abnormalities as well as lung defects (150). Most of them
die perinatally; however, some survive to adulthood, constitut-

Fig. 2. History of the discovery and characterization of megalin, cubilin, and the folate receptor (FR) in the kidney. Modified from Ref. 27 with permission from
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 proteinuria and have been described (Table 1), which many may be filtered in the renal glomerulus and reabsorbed by cubilin-mediated endocytosis, including albumin, DBP, and α-amylase (12). Thus RAP-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 to show 68% homology, whereas almost 100% homology is observed between human FRα and soluble human milk FBP lacking the GPI anchor. FRs also reveal some homology with riboflavin-binding protein. The GPI-anchored FRs are attached to the membrane at the amidated COOH-terminal serine or asparagine, through a phosphodiester linkage of phosphoethanolamine to a tri-mannosyl glucosamine core. The glucosamine is linked to phosphatidylinositol anchored to the cell membrane.

![Diagram of megalin structure](http://ajprenal.physiology.org/)

Fig. 3. Schematic representation of the structure of megalin, cubilin, AMN, and the FR. Megalin is an 4,600-amino acid transmembrane protein with a nonglycosylated molecular mass of 517 kDa. The larger extracellular domain is composed of 4 cysteine-rich clusters of LDL receptor type A repeats constituting the ligand binding regions, separated and followed by a total of 17 EGF-type repeats and 8 spacer regions containing YWTD repeats. The single transmembrane domain is followed by the cytoplasmic tail containing 2 NPXY sequences, 1 VENQNY sequence, involved in apical sorting (137), and several Src homology 3 and 1 Src homology 2 recognition sites possibly involved in signal transduction. Cubilin is a 3,600-amino acid protein with no transmembrane domain and a nonglycosylated molecular mass of 400 kDa. The extracellular domain is composed of 27 bone morphogenic protein-1 (CUB) domains responsible for interaction with a multiple of other proteins. The CUB domains are preceded by a stretch of 110 amino acids followed by 8 EGF-type repeats. The NH2-terminal region contains a potential palmitoylation site and an amphipatic α-helix structure with some similarity to the lipid-binding regions of apolipoproteins. AMN is a 434-amino acid, single-transmembrane protein with a nonglycosylated molecular mass of 46 kDa. It has no known closely related proteins, but a cysteine-rich stretch of 70 amino acids in the extra-cellular domain is similar to modules present in a small group of proteins serving as bone morphogenic protein inhibitors. GPI-anchored FR isoforms are 210-amino acid glycoproteins with a nonglycosylated molecular mass of 25 kDa. Human FRα and FRβ show 68% homology, whereas almost 100% homology is observed between human FRα and soluble human milk FBP lacking the GPI anchor. FRs also reveal some homology with riboflavin-binding protein. The GPI-anchored FRs are attached to the membrane at the amidated COOH-terminal serine or asparagine, through a phosphodiester linkage of phosphoethanolamine to a tri-mannosyl glucosamine core. The glucosamine is linked to phosphatidylinositol anchored to the cell membrane.

![Diagram of megalin structure](http://ajprenal.physiology.org/)

Little is known about the regulation of megalin gene expression in vivo; however, receptor-associated protein (RAP), a 45-kDa protein modulating posttranslation 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 an 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 (155). 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. 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. 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.
2 (154). The NH₂-terminal region contains a furin-cleavage site, a potential cysteine palmitoylation site, and a putative amphipathic helix structure similar to the lipid-binding regions of apolipoproteins (74).

RENAL EXPRESSION. Cubilin is highly expressed in the renal proximal tubule (Fig. 4) (119). As with megalin, immunoreactive cubilin can also be identified in lysosomes (126). Although the extrarenal 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 coelute 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 patients with inherited vitamin B₁₂ deficiency syndrome characterized by defective intestinal absorption of IF-B₁₂ (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-B₁₂ 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 Imerlund-Gräsbeck syndrome, it may be 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-B₁₂-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). 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 (Kd ~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).

RENSAL 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 B₁₂ 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, Ca²⁺-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,
including DBP, immunoglobulin light chains, albumin, and RAP (Table 1). Thus in epithelia coexpressing megalin and cubilin, including kidney proximal tubule, megalin and cubilin appear to act in concert, mediating endocytosis of the same ligands. Megalin also binds milk FBP (14), providing a mechanism for the uptake of FBP-bound folate. Soluble milk FBP structurally resembles GPI-linked FRs (60, 81) coexpressed with megalin in the luminal plasma membranes of kidney proximal tubule and other epithelia, i.e., the choroid plexus and yolk sac (28). No direct interaction between megalin and GPI-linked FRs in the plasma membrane has been demonstrated; however, it has previously been shown that the related LRP mediates internalization of the GPI-anchored urokinase receptor (33), indicating that a similar interaction between megalin and FR is possible. This may lead to the hypothesis that megalin is involved in intracellular translocation of FRs, possibly regulating luminal expression of membrane FR by internalization and degradation.

Proximal Tubule Uptake of Folate and Vitamin B\textsubscript{12}

Proximal tubule uptakes of both folate and vitamin B\textsubscript{12} 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 B\textsubscript{12} 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 B\textsubscript{12}. Megalin is essential for the proximal tubule reabsorption of filtered TC-B\textsubscript{12} (13), mediating endocytosis of TC-B\textsubscript{12} (90, 104). Megalin and TC colocalize within the endocytic apparatus of rabbit kidney proximal tubule cells (142). TC-B\textsubscript{12} binds to megalin with an estimated affinity (\(K_d\)) of \(~183\) nM when analyzed by surface plasmon resonance (SPR) analysis, also showing a possible second binding site (\(K_d\) \(~1.4\) \(\mu\)M). 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-B\textsubscript{12} was established using megalin knockout mice (13). These revealed increased urinary excretion of B\textsubscript{12} and a 28-fold increase in renal B\textsubscript{12} clearance, along with a 4-fold decrease in the B\textsubscript{12} 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-B\textsubscript{12} 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-B\textsubscript{12} uptake is supported by the low tissue B\textsubscript{12} and apparent vitamin B\textsubscript{12} deficiency developing in rabbits following injection with an antiserum against this receptor (16) and by the decrease in renal uptake of orally administered labeled B\textsubscript{12} in adrenalec- tomized rats (17). It was recently suggested that this ubiquitous TC-B\textsubscript{12} receptor binds to megalin and that this binding is associated with increased binding of TC-B\textsubscript{12} to the purified receptors (153). It was also shown that the immunization of rabbits with megalin was associated with decreased expression of the TC-B\textsubscript{12} 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-B\textsubscript{12} receptor awaits further clarification. Evidence suggests that the major mechanism for renal accumulation of vitamin B\textsubscript{12} is by tubular reabsorption of filtered TC-B\textsubscript{12}. Thus the 10–90% distribution of the 62-kDa TC-B\textsubscript{12} 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 B\textsubscript{12} injected during vitamin depletion as well as during vitamin load fits a model of cellular TC-B\textsubscript{12} uptake involving two distinct receptors: a possibly regulated ubiquitous TC-B\textsubscript{12} receptor mediating saturable cellular uptake, and a luminal renal tubule receptor mediating constitutive high-capacity reabsorption of filtered TC-B\textsubscript{12} (7, 90).

Although both megalin and cubilin mediate endocytic uptake of vitamin B\textsubscript{12}, and both are heavily expressed in proximal tubule epithelial cells, a role for cubilin in kidney B\textsubscript{12} uptake has not been established. Cubilin purified from kidney binds IF-bound B\textsubscript{12}, and uptake of IF-B\textsubscript{12} 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 B\textsubscript{12} filtered in complex with IF, the significance of cubilin for renal uptake of B\textsubscript{12} 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 FR\textsubscript{a}) 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 FR\textsubscript{B}) 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 transtubular 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 [1H]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 \( \mu M \). 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 \( \mu g \) 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 \( K_A \) of ~0.6 \( \mu m \) (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 \( B_{12} \) 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 \( B_{12} \) accumulates and is only slowly released (79, 104). Studies on the distribution of a single oral or parenteral dose of labeled \( B_{12} \) 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). Accumulated vitamin B12 may accumulate 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 possibly 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 in the cell before secretion or are secreted separately remains to be established.
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.

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