Endocytic Receptors in the Renal Proximal Tubule

Protein reabsorption is a predominant feature of the renal proximal tubule. Animal studies show that the ability to rescue plasma proteins relies on the endocytic receptors megalin and cubilin. Recently, studies of patients with syndromes caused by dysfunctional receptors have supported the importance of these for protein clearance of human ultrafiltrate. This review focuses on the molecular biology and physiology of the receptors and their involvement in renal pathological conditions.

Human urine is almost devoid of protein, although the glomeruli filter significant amounts. The cutoff limit for glomerular filtration of circulating macromolecules is \( \sim 60 \text{ kDa} \), although larger proteins like albumin (66 kDa) and transferrin (81 kDa) also are filtered to a limited extent. In the normal kidney, reabsorption of filtered proteins occurs almost exclusively by receptor-mediated endocytosis in the proximal tubule. The apical endocytic apparatus is very elaborate in the first two segments of the proximal tubule (44), consisting of coated pits, coated vesicles, endosomes, lysosomes, and dense apical tubules responsible for membrane and receptor recycling from endosomes to the apical plasma membrane (35). During the past 20 years, it has become clear that this efficient reabsorption of proteins is mediated by two large membrane receptors, megalin (90) and cubilin (150). These receptors bind a variety of filtered ligands with varying affinities, and due to the great abundance and efficient recycling of receptors at the apical plasma membrane, the reabsorption is very efficient. In addition to the simple removal of urinary proteins providing a nontoxic, protein-free milieu in more distal parts of the nephron and the collecting ducts, this process serves a number of other important physiological functions. The reabsorption recovers essential substances that otherwise would be lost in the urine, notably vitamins and different trace elements bound to their carrier proteins. Although proteins are degraded in lysosomes, vitamins and trace elements are returned to the circulation by transport across the basolateral membrane. In addition, reabsorbed substances may be metabolized within the proximal tubule cells as illustrated by the important, renal activation of endocytosed 25-OH-vitamin D3 (128, 129). Transcytosis of intact protein, however, appears not to take place to any significant extent in intact proximal tubules in vivo as recently reviewed in more detail (44).

In this review we will describe the structure and localization of the receptors and ligand-receptor interactions in more details and we will review different types of extra-renal based, glomerular and tubular proteinuria, in which the receptors are involved.

Structure and Expression of Megalin, Cubilin, and Amnionless

Megalin

Megalin (FIGURE 1) was identified 30 years ago by Farquhar and Kerjaschki (90, 91). Cloning and sequencing of the megalin encoding gene LRP2 uncovered a giant, glycosylated protein (600 kDa, 4,655 amino acids) with similarities to endocytic receptors of the LDL receptor family (78, 146, 152). Several predominantly murine studies support the endocytic function of megalin, and, in the proximal tubule, megalin drives the reabsorption of nearly all filtered plasma proteins in cooperation with the receptor protein cubilin (36, 43). Thus megalin serves as a multispecific clearance receptor, and the structural basis for this resides in the large, extracellular domain consisting of four clusters of ligand binding, cysteine-rich complement-type repeats (57, 78, 147, 152). The clusters contain 7–11 complement-type repeats, each consisting of \( \sim 40 \text{ amino acids} \), and a more detailed analysis of the 12th repeat of megalin revealed that, similar to the LDL receptor, it holds three disulfide bridges and a COOH-terminal calcium cage (12, 57, 184). The binding clusters are separated by YWTD and EGF repeats, which are involved in the dissociation of ligands in acidic compartments and in the recycling of the receptor (50). Megalin contains a single, transmembrane domain (23 amino acids), which is flanked on the extracellular side by an EGF-like module and on the intracellular side of the COOH-terminal cytoplasmic tail of 209 amino acids.

Megalin is present in the apical membranes of absorptive epithelia and is heavily expressed in the...
proximal tubule (FIGURE 2) located on the brush border and endocytic vesicles for clearance of the filtrate and in dense apical tubules for recycling to the apical membrane (33, 37, 42, 92). A minor fraction of megalin is also present in lysosomes probably when determined for degradation (42). Since megalin was initially identified in rat podocytes as the antigen-inducing formation of immune deposits in a rat model of Heymann nephritis, it was expected to be present in podocytes from other species. It has so far not been detected in mouse, but recently it was identified in human podocytes, where the receptor was shown to be endocytic active (143).

Modules in the cytoplasmic domain of megalin regulate receptor trafficking and endocytosis. The two NPXY motifs sequester protein complexes consisting of, among others, clathrin, AP-2, Dab2, and ARH, which are involved in coated pit formation (93, 124, 132, 154). The importance of the recruitment of Dab2 for the endocytic process is suggested by the development of mild proteinuria in Dab2 knockout mice (120). Endocytosis requires involvement of cytosolic and cytoskeletal components, and the megalin-driven process has been associated with nonmuscle myosin heavy chain IIA, actin, and myosin VI through interaction with Dab2 and another adaptor protein, GIPC (80, 112, 123). Both myosin VI and GIPC knockout mice have albuminuria supporting the functional relevance of these interactions (65, 123). Interestingly, common variants in the MYH9 locus (encoding nonmuscle myosin heavy chain IIA) have recently been shown to be associated with FSGS and non-diabetic kidney disease in African-American individuals (88, 96), the Dab2 locus to CKD (97), and a connection between LRP2 and alcohol to hyperuricemia (71).

The NPXY-like motif has been reported to direct megalin to the apical membrane of the cell (167), which also involves receptor-associated protein (RAP) binding to the extracellular megalin domain and serving as a chaperone (17, 28).

The megalin cytoplasmic tail holds several phosphorylation and additional protein interaction motifs, and a number of interacting proteins have been discovered (66, 78, 104, 139). However, the function, cooperation, and tissue specificity of most reported cytoplasmic binding molecules remains to be established. One of the phosphorylation sites, a PPPSP motif, is constitutively phosphorylated and shown to regulate receptor recycling (188).

**Cubilin**

The almost complete clearance of proteins from the primary filtrate by megalin-driven endocytosis is accomplished in cooperation with the receptor cubilin. Cubilin is co-expressed with megalin in the apical endocytic compartments of the proximal tubule (FIGURE 2) (150, 151, 158) and was also recently demonstrated in rat and human podocytes (142). Cubilin was originally identified as the intestinal intrinsic factor $\text{B}_{12}$ receptor (158, 159) and shown to be a 460-kDa glycosylated protein.
(FIGURE 1) composed of 27 COOH-terminal CUB domains [complement c1r/c1s, uegf (epidermal growth factor-related sea urchin protein) and bone morphogenic protein 1] (102, 117). The numerous CUB domains are ligand binding, and each consists of ~120 residues also identified in several other proteins such as BMP-1, tolloid, and TSG6 (6, 22, 103, 187). Cubilin-ligand interactions are Ca<sup>2+</sup>-dependent, similar to the LDL receptor family (6). The NH<sub>2</sub>-terminal part of cubilin consists of eight epidermal growth factor repeats and a 110-amino acid stretch (102, 117).

Since cubilin lacks a transmembrane and a cytoplasmic domain, it is obliged to interact with other membrane proteins for endocytosis. In the proximal tubule, it is believed to interact with megalin, forming a two-receptor complex, with megalin directing internalization of the complex and bound ligands. This notion is based on the co-localization of the two receptors, a direct interaction of cubilin with megalin through CUB domain 12–17 and 22–27 as well as through the NH<sub>2</sub>-terminus, and the observation that megalin knockout animals fail to reabsorb endogenous cubilin ligands at any detectable level (2, 4, 117, 187).

**Ammionless**

The normal function of cubilin is also dependent on the 38- to 50-kDa, single transmembrane protein amnionless (AMN) (FIGURE 1) (59, 168). In the proximal tubule, AMN co-localizes with cubilin and is essential for the trafficking of cubilin to the apical membrane, as evidenced by intracellular cubilin retention in proximal tubules of dogs with AMN mutations, causing Imerslund-Gräsbeck syndrome in humans, and in AMN-deficient mice (2, 59, 75, 166). The opposite dependence also holds true in the proximal tubule, as evident from studies in both cubilin-deficient mice and patients (4, 165). Interactions between AMN and the EGF domains in cubilin are believed to be responsible for this interdependence (48, 59). AMN has an extracellular domain of 70 amino acids with the only characteristic domain being a cysteine-rich stretch (85). AMN contains a cytoplasmic tail including two NPXY motifs, and in cell cultures lacking megalin, cubilin is endocytosed, probably relying on these endocytic motifs in AMN (59, 85). In line with this observation, intestinal absence of megalin in Donnai-Barrow patients apparently does not result in defect intestinal cubilin endocytosis and B<sub>12</sub> deficiency, as observed in individuals with dysfunctional cubilin or AMN (Imerslund-Gräsbeck disease) (see also later), suggesting that AMN directs cubilin endocytosis in the intestine. As mentioned above, this cooperation is unable, for unknown reasons, to uphold cubilin endocytosis in the proximal tubule.

However, if AMN contributes to cubilin endocytosis also when cubilin is associated to megalin is difficult to exclude, since studies of cubilin without AMN are difficult to perform due the interdependent apical sorting of AMN and cubilin.

**FIGURE 2. Immunofluorescence for megalin and cubilin of paraffin sections**

Immunofluorescence for megalin and cubilin of paraffin sections from mouse kidney from the renal capsule (top) through the cortex and the outer stripe of the outer medulla. Only proximal tubules, including proximal tubule cells constituting part of Bowman’s capsule of the glomeruli (G) are labeled. A, arteries; ISOM, inner stripe of outer medulla. Bar = 100 μm. Figure was adapted from Ref. 41, with permission from Springer Verlag.
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Function of Megalin and Cubilin in the Proximal Tubule

Ligands

Megalin binds and mediates the endocytosis of a large and highly diverse group of ligands, including plasma proteins, peptides, enzymes, vitamin-binding proteins, hormones, and hormone-binding proteins, as well as drugs and toxins. Some of the ligands are shared with cubilin, whereas others are specific for either megalin or cubilin (Table 1).

A ligand of particular interest is albumin, the most abundant plasma protein and most widely used urinary marker of kidney disease. Albumin has been shown to bind both cubilin (14) and megalin (49). Tubular uptake of albumin is markedly decreased in conditional cubilin-deficient mice (4, 181), in dogs with cubilin dysfunction due to mutations in \textit{AMN} (14), and in humans with mutations in the cubilin gene \textit{CUBN} (165). The virtually complete inhibition of proximal tubular uptake of albumin in megalin-, cubilin-, and double-KO mice (4, 181), indicates that the main role of megalin in albumin reabsorption is to drive the internalization of cubilin-albumin complexes. This is further supported by the identification of a single-nucleotide polymorphism in the megalin-binding region of cubilin that is associated with microalbuminuria in both the general population and diabetic individuals (20). Recent investigations of albumin handling in megalin-/cubilin-deficient mice determined the urinary excretion of albumin in the absence of both megalin and cubilin to be 1.45 ± 0.54 mg/day, which translates to an albumin concentration in the glomerular ultrafiltrate of ~4 μg/ml (181). This value is in agreement with micropuncture measurements made in rats (172) but is 10–100 times lower than the values measured by two-photon microscopy (148, 149, 153). Also, no albumin degradation occurs in proximal tubular cells in the absence of megalin/cubilin, demonstrating that megalin-/cubilin-mediated endocytosis is required for the intracellular degradation of albumin (FIGURE 3) (180). It has been proposed that degraded albumin is released as fragments into the tubular lumen, resulting in the excretion of large amounts of albumin fragments in the urine (69, 135). However, the urinary excretion of albumin fragments is unaltered by megalin/cubilin deficiency, suggesting that the source of the urinary albumin fragments is independent of megalin and cubilin (180). This supports the concept that proximal tubule endocytic uptake of albumin leads to its accumulation and degradation in the lysosomal compartment (24, 113, 138, 160), which results in the formation of free amino acids that are released into the circulation (FIGURE 4).

Vitamin Metabolism

Megalin and cubilin play a crucial role in the tubular retrieval of several plasma carriers for vitamins and nutrients (FIGURE 4). These include the vitamin D-binding protein (DBP) (128, 129), retinol-binding protein (RBP) (40), folate-binding protein (19), and transcobalamin-B_{12} complex (TC-B_{12}) (18, 115, 127), which are plasma transporters for vitamin D, vitamin A, folate, and vitamin B_{12}, respectively. Consistent with this, excretion of the carriers in megalin knock-out mice resulted in concomitant loss of the bound vitamins (18, 40, 45, 77, 128, 144). Following reabsorption and release from carrier proteins in the endosomal/lysosomal compartment, vitamins and nutrients are believed to be transported across the basolateral membrane and thereby returned to...
the circulation. In addition, at least in rodents, the kidney and megalin also plays an important role for accumulation and storage of vitamin B₁₂ (13, 18). Furthermore, megalin- and cubilin-mediated reabsorption of filtered 25-OH-vitamin D is essential for the renal hydroxylation of the vitamin into the active 1,25-(OH)₂ vitamin D₃. Accordingly, megalin-deficient mice have low serum levels of 1,25-(OH)₂ vitamin D₃, disturbed calcium homeostasis, and decreased bone mineralization (105, 128), similar to observations in dogs with cubulin dysfunction due to mutations in AMN (129).

**Signaling**

In addition to controlling endocytosis, the interactions between megalin and scaffold proteins, usually mediated through cytosolic adaptors, have been suggested to function in intracellular communication and signal transduction (66). Also, megalin has been shown to undergo regulated, intramembranous proteolysis similar to other large transmembrane receptors, such as the Notch receptor, where the intracellular domain (ICD) is released into the cytoplasm by γ-secretase activity (191). However, in contrast to the Notch receptor where the role of the ICD in gene regulation is well characterized (25), the role of intramembranous proteolysis for most receptors remains unresolved in vivo. Megalin ICD overexpression in cell culture in vitro has been shown to result in a downregulation of megalin and NHE3 transcripts (109). However, recent in vivo studies using a mouse model expressing the megalin ICD under control of the endogenous megalin promoter found no distinct effects on renal proximal tubular function (34). Accordingly, it appears less likely that release of the megalin ICD, triggered by ligand binding, may reduce receptor activity and protect proximal tubules from harmful effects of protein overload, as has been proposed (11).

**Genetic Disorders of Megalin and Cubulin in Humans**

Rare syndromes associated with genetic defects of megalin and cubulin have been reported, providing important information on the function of these receptors in humans. Mutations of LRP2 have been identified in patients with Donnai-Barrow Syndrome (DB) (54) and Facio-Oculo-Acustico-Renal (FOAR) Syndrome (79) (DB/FOAR, OMIM no. 222448) (87), suggesting the disorders to be allelic (87). The DB/FOAR syndrome is an exceptionally rare, autosomal recessive inherited disorder (141). Consistent with the expression of megalin in several tissues, DB/FOAR patients present with variable and multiple developmental anomalies including hypertelorism, large anterior fontanelle, agenesis of the corpus callosum, congenital diaphragmatic hernia, omphalocele/umbilical hernia, a number of characteristic facial features, as well as functional deficits including sensorineural hearing loss, high myopia, and low-molecular-weight proteinuria (141). In line with observations in megalin-deficient mice (18, 40, 45, 106, 128), increased urinary excretion of megalin ligands (FIGURE 5), vitamin D-binding protein, retinol-binding protein, and albumin have been consistently reported in DB/FOAR patients (87, 122). The phenotype of these patients is, however, complex, and so far the long-term implications of renal megalin deficiency in humans have not been investigated.

Mutations of the cubulin encoding gene (CUBN) or the amnionless encoding gene (AMN) (168) have been identified in patients with Imerslund-Gräsbeck Syndrome, also known as Megaloblastic Anemia 1 (IGS or MGA1, OMIM no. 261100) (3), and is characterized by megaloblastic anemia and low-molecular-weight proteinuria (67, 84). Both genetic defects are inherited in an autosomal recessive manner and result in intestinal malabsorption of vitamin B₁₂ and symptoms associated with B₁₂ deficiency (68). The vitamin B₁₂ deficiency is easily corrected with repeated parental injections of vitamin B₁₂, alleviating most symptoms except for the low-molecular weight proteinuria (68).

Most IGS patients demonstrate increased urinary excretion of cubulin ligands (FIGURE 5) including albumin (178), transferrin (101), VDBP (129), and apo A-I (100), and analyses of renal biopsy material from a cubulin-deficient patient recently identified the molecular background for this as proximal tubular dysfunction of cubulin (165). The proteinuria is generally believed to be "benign" with preservation of kidney function over time (68), although minor, glomerular changes have been described (26, 47). In contrast to most reported cases (23, 26, 27, 47, 67, 74, 84, 107, 125, 178), patients harboring a particular CUBN
Megalin- and cubilin-mediated uptake of vitamin carrier protein complexes and albumin in renal proximal tubule

Following receptor-mediated endocytosis via apical coated pits, the complexes accumulate in lysosomes for degradation of the proteins, whereas the receptors including AMN recycle to the apical plasma membrane via dense apical tubules. As illustrated here and detailed in the text, megalin mediates the uptake of cubilin and its ligands. Whether the two receptors are constitutively associated in the plasma membrane and remain associated during recycling in dense apical tubules is not known. Whereas some vitamin carriers such as TC and RBP apparently bind exclusively to megalin, others such as DBP bind with similar affinity to both megalin and cubilin, and albumin probably with highest affinity to cubilin. The intracellular processing of the vitamins include modifications such as hydroxylation of 25(OH)D3 to 1,25-(OH)2D3 and metabolism of B12. The mechanisms for the cellular release of the vitamins remain to be clarified.
missense mutation (P1297L, also known as FM1) (3, 100, 129) affecting the intrinsic factor binding site generally do not present with an overt tubular proteinuria, suggesting that the proteinuria is mutation-type dependent.

Recently, a single base pair deletion of CUBN exon 53 (~CUB domain 20) described two siblings presenting with albuminuria without megaloblastic anaemia (136). In addition, as previously mentioned, a missense variation in CUB domain 22 was associated with albuminuria in populations of European and African ancestry (20). Combined, this hereby suggests that albuminuria may in some cases be a result of genetic variation of CUBN causing differences in cubilin function and consequently defective tubular albumin reabsorption.

A number of other genetic disorders affecting proximal tubule cubilin and megalin expression and function have been described. These include Dent’s disease 1 and 2 (OMIM no. 300009 and no. 300555) (52) as well as Lowes syndrome (OMIM no. 309000) (111). In these conditions, expression and trafficking of the receptors is affected (38, 55, 140), leading to low-molecular weight proteinuria similar to the proteinuria observed in the DB/FOAR patients, although notably milder.

**Megalin and Cubulin in Acquired Renal Diseases**

Since the important physiological role of proximal tubule receptor-mediated endocytosis has been established, focus has been attracted to the possible involvement of megalin and cubilin in renal diseases. This may be perceived in different ways: 1) receptor function or dysfunction as a cause of disease and 2) receptor dysfunction as a marker or consequence of disease. Although it may be difficult to separate these, especially in the study of human diseases, the appreciation of the different mechanisms is important. Receptor function or dysfunction as a cause of disease essentially would suggest interventions targeted at modifying receptor function.

**Receptor Function or Dysfunction as a Cause of Disease**

Megalin- and/or cubilin-mediated uptake of nephrotoxic substances has been well established. These
include gentamicin and aprotinin (73, 116, 155), radiolabeled somatostatin analogs (8, 51), cadmium (95, 185), myoglobin (60), and hemoglobin (61) causing pigment cast nephropathy, as well as light chains disease (9, 15, 108). Inhibitors of megalin have been explored in the prevention of nephrotoxicity from gentamicin (179) and somatostatin analogs (175).

Excessive uptake of proteins (FIGURE 5) has been implicated in the progression of chronic kidney disease (1). In vitro studies have established a number of pathways by which albumin and other filtered proteins may activate cellular pathways in proximal tubule cells leading to apoptosis, endoplasmic reticulum stress, interstitial inflammation and fibrosis, and possibly epithelial-mesenchymal transformation (1), eventually leading to accelerated nephron loss. In vitro observations using siRNA inhibition of megalin expression have furthermore suggested a direct role of this receptor in albumin-induced activation of proximal tubule tubular synthesis of components of the renin angiotensin system (RAS) (30). Tubular activation of RAS is believed to be associated with the development of hypertension, albuminuria, tubular apoptosis, and tubulointerstitial fibrosis (63). A role of megalin in the regulation of apoptosis has also been proposed based on in vitro observations showing interaction between megalin and the phosphokine nase PKB in the luminal membranes (31). Albumin overload in vitro has been associated with a decrease in megalin levels leading to reduced PKB activity and apoptosis (31). A role of megalin and cubilin in protein overload-induced tubulopathy and interstitial inflammation and fibrosis is, however, not definite (53). By using conditional megalin knockout mice, the role of megalin for the progression of renal disease was evaluated in two models of kidney injury induced either by injection of anti-glomerular basement membrane antibodies (170) or induction of podocyte dysfunction (121). The results are somewhat inconsistent, and no conclusive evidence for a role of megalin or cubilin has been identified; however, both studies showed an upregulation of some inflammatory or fibrotic markers in predominantly megalin-positive cells, suggesting that megalin deficiency may prevent activation of these pathways, whereas divergent findings were reported regarding apoptosis. In both studies, mice were analyzed only 18 and 10 days, respectively, after the induction of renal damage, suggesting that the observed changes more likely reflect acute injury rather than the slow progression of renal disease in humans with proteinuria. Recently, adenoviral delivery of antisense RNA leading to partial knockdown of cubilin was shown to protect against adriamycin-induced glomerulosclerosis and tubulointerstitial damage in rats despite higher levels of albuminuria (110). For unknown reasons, knockdown of cubilin was also associated with reduced levels of megalin mRNA. Further studies are needed to establish the consequences of increased megalin- and cubilin-mediated protein uptake for the progression of proteinuric chronic kidney disease.

Receptor Dysfunction as a Marker of Disease

Proteinuria probably remains one of the most sensitive markers of renal disease; however, in most cases, it is not clarified to what extent this reflects defects in receptor-mediated protein reabsorption. Several urine biomarkers of acute and chronic kidney disease, such as albumin, NGAL, cystatin C, and L-FABP have been identified as ligands for megalin and/or cubilin (4, 14, 49, 83, 89, 137, 189), suggesting that defective protein uptake may play a role for the increased urinary excretion of these; however, little is known about megalin and cubilin function in these conditions.

Changes in receptor expression have been reported in animal models of acute and chronic renal disease, including lipopolysaccharide-induced endotoxemia (156), ischemia-reperfusion kidney injury (156, 177), shiga toxin-induced nephropathy (130), hypertension and chronic kidney disease by overexpression of renin (82, 182), diabetes (7, 174), and the remnant kidney model of chronic kidney disease (94). Increased urinary excretion of megalin, megalin fragments, and cubilin (FIGURE 5) have been identified in models of Alport syndrome (176) and experimental as well as human diabetes (58, 131, 171, 173). In most conditions, a reduction in megalin expression has been reported; however, e.g., in models of chronic kidney disease, observations are not consistent (82, 94), and, although receptor expression may be affected in various renal conditions, it is currently not clear whether the changes reflect functional changes and specific pathogenic mechanisms. In experimental diabetes, blockade of the renin-angiotensin system is suggested to restore normal megalin expression associated with a decrease in albuminuria (174), indicating that megalin function is a potential target in current anti-proteinuric therapy by RAS inhibition. This may be supported by the observation that megalin expression is reduced in vitro by activation of tubular angiotensin II type 1A receptors (81). Whether similar mechanisms are important in human diabetic kidney disease has not been established.

In conclusion, megalin and cubilin interact in the proximal tubule to mediate the uptake of virtually all filtered proteins. This process is important not only for the clearance of proteins from the ultrafiltrate but is also essential for the renal metabolism of vitamins and hormones as well as for
the tubular uptake of nephrotoxic substances. Receptor dysfunction has been identified in rare genetic disorders but may also be associated with more common, acquired forms of renal diseases. Whether this dysfunction is of importance for the development and progression of these diseases remains to be established.

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author contributions: E.I.C., H.B., T.S., K.W., and R.N.

No conflicts of interest, financial or otherwise, are declared by the author(s).


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