Full Review

From bowel to kidneys: the role of cubilin in physiology and disease

Erik I. Christensen, Rikke Nielsen and Henrik Birn

Section of Cell Biology, Department of Biomedicine, Faculty of Health Sciences, Aarhus University, Aarhus, Denmark

Correspondence and offprint requests to: Erik Ilsø Christensen; E-mail: eic@ana.au.dk

Abstract

Cubilin is a large endocytic receptor serving such diverse functions as the intestinal absorption of the intrinsic factor–B12 complex and the renal proximal tubule reabsorption of filtered proteins including albumin, transferrin, vitamin D-binding protein and other important plasma carriers. Cubilin is a structurally unique, peripheral membrane protein, which depends on the membrane protein amnionless (AMN) for correct apical translocation. In addition, AMN appears important for efficient internalization of intrinsic factor–B12 in the intestine, whereas in the proximal tubule cubilin interacts with another endocytic receptor, megalin, for effective reabsorption. The importance of cubilin has been demonstrated in several animal models of cubilin deficiency as well as in a variety of human diseases. Recent demonstration of cubilin in podocytes from various species awaits further clarification with respect to the functional role as well as its role in pathology.

Keywords: albuminuria; Imerslund-Gräsbeck syndrome; low-molecular-weight proteinuria; vitamin B12; receptor-mediated endocytosis

Introduction

Receptor-mediated endocytosis is a general process by which cells can selectively and actively internalize biological substances. It is essential to normal life and occurs in almost all tissues. It is tightly regulated by intracellular mechanisms involving pathways common to many different cell types. In some cases, receptors have evolved serving very different physiological functions in different tissues. The endocytic receptor cubilin has very important, yet very different functions, mediating the uptake of intrinsic factor–vitamin B12 in the intestine as well as the uptake of filtered proteins, including albumin, in the kidneys (Figure 1). Since then, the importance of cubilin in normal renal physiology has been established and new evidence suggests a role of cubilin in the development and progression of chronic kidney disease. In this paper, the fascinating history leading to the identification of cubilin will be summarized followed by a review of current knowledge on the structure, function and pathophysiological role of this receptor.

In 1926 the therapeutic effect of orally administered liver on pernicious anaemia was demonstrated [1], and the studies by Castle et al. [2] showed that a gastric substance (intrinsic factor, IF) acted together with the extrinsic factor, vitamin B12, to prevent this disease. Receptor-mediated uptake of IF–vitamin B12 complexes in the small intestine was suggested by Herbert in 1959 [3] and several groups attempted to purify the receptor (reviewed by Seetharam and Alpers in 1982 [4]). In 1960 a much more rare, inherited disease characterized by juvenile onset and intestinal malabsorption of vitamin B12 was described simultaneously by Imerslund [5] and Gräsbeck et al. [6]. This disease, known as Imerslund-Gräsbeck syndrome (IGS), is often associated with proteinuria and it was noted that the anaemia was corrected by intramuscular injections of the vitamin B12; however, the proteinuria was not.

In the 1980s Verroust et al. [7–9] purified and characterized a protein (gp280) localized in the clathrin-coated luminal membrane domains in renal proximal tubules and yolk sac epithelia. These observations indicated that gp280 might be an endocytic receptor. Simultaneously, Seetharam et al. [10] isolated an IF–B12-binding, renal brush border protein that appeared to be identical to the small intestinal IF–B12 receptor and later shown to be a 460-kDa protein identical to gp280 [11, 12]. The protein was cloned from rats [13] and humans [14] and shown to be a peripheral membrane protein, named cubilin.

In 1999 mutations in the cubilin gene CUBN were identified as the cause of IGS in a group of Finnish patients [15]. Prior to this, Fyfe et al. [16] described a family of giant schnauzers with an inborn, intestinal IF–B12 malabsorption similar to the human IGS [17]; however, with no apparent mutations in cubilin [18]. It was shown that these dogs had a reduced brush border expression of cubilin [16]. In 2003 Tanner et al. [19] identified mutations in the AMN gene encoding the protein amnionless (AMN) in Norwegian and Tunisian IGS families. Similarly, the dogs were shown to harbour a mutation in AMN [20, 21] These observations led to the demonstration that AMN and cubilin form a complex
Cubam and that AMN is essential for normal apical expression of cubilin [22], see also Coudroy et al [23].

Cubilin structure

The cloning of cubilin revealed a protein different from all other known receptors at the time [13, 14]. The name of the receptor refers to the 27 CUB [complement c1r/C1s, Uegf (epidermal growth factor-related sea urchin protein) and bone morphogenetic protein 1 (BMP)] domains found in the C-terminal part of the protein (Figure 2). They represent ~88% of the glycosylated 460-kDa-sized protein each composed of ~110 amino acids [13, 14]. The CUB domains are also identified in several developmental regulated proteins such as BMP-1, tolloid and TSG6 [24]. In the N-terminal region eight Ca\textsuperscript{2+}+-binding epidermal growth factor repeats and a terminal region involved in trimerization of the protein are situated [13, 14]. In cubilin the CUB modules act as ligand-binding domains and the intrinsic factor–B12 complex binds domains 5–8 [25, 26], which has recently been crystallized [27].

Cubilin harbours no transmembrane domain and it is thus dependent on additional factors for membrane anchoring and for endocytosis of the receptor-bound ligands. Initially, cellular translocation of cubilin was believed to rely solely on megalin, constituting an additional, multi-ligand receptor expressed in the proximal tubule [28]. This was based on subcellular co-localization of the receptors in the proximal tubule, and a direct interaction of the receptors through CUB domains 12–17, 22–27 and the N-terminus of cubilin [8, 11, 13, 26, 29, 30]. Furthermore, megalin knockout mice as well as megalin-deficient patients (DB/FOAR patients; OMIM # 222448) fail to reabsorb endogenous cubilin ligands [31, 32] supporting the importance of megalin for normal endocytic activity of cubilin in the proximal tubule. The cytoplasmic tail of megalin contains two endocytic motifs believed to drive internalization of the complex [28]. In addition, megalin is a multi-ligand receptor harbouring an extensive extracellular domain which includes four binding clusters responsible for binding multiple, diverse ligands [28].

Fig. 1. The physiological role of cubilin as an endocytic receptor in the intestinal and renal proximal tubule epithelial cells.

Fig. 2. Graphic presentation of cubilin and AMN. The illustration depicts known domains of the receptors. The CUB domains are ligand binding, but as cubilin is a peripheral membrane protein lacking intracellular motifs it is dependent on other proteins for endocytosis. In the intestine AMN is believed to assist cubilin in absorption of IF–B12, whereas in the proximal tubule endocytosis by this complex is limited. Here megalin (not shown) is required for internalization of cubilin ligands. AMN is furthermore needed for membrane localization of cubilin.
In addition to the endocytic apparatus of the small intestine [33], and the proximal tubule [7–9], cubilin is also expressed in podocytes [34], placenta [35], inner ear [36] and the visceral yolk sac [8].

Amnionless

As mentioned, cubilin was shown to associate with AMN [22], a 38–50-kDa protein essential for amnion and primitive streak development in mice [19, 37, 38]. AMN is a transmembrane protein with a cytoplasmic tail containing two NPXY motifs and a cystein-rich stretch as the only known characteristic feature of the extracellular domain (Figure 2) [22, 38]. The domain shares similarities with cysteine-rich domains found in bone BMP-binding proteins and may play a role in modulation of BMP signaling. Cubilin and AMN, in complex also known as CUBAM [22], co-localize closely in the kidneys [22], intestine [19] and yolk sac of mice [38] and AMN is essential during biosynthesis and trafficking of cubilin. The two proteins assemble, through the EGF domains in cubilin, early in the translational machinery, and mutations in the AMN gene in both mice and dogs cause retention of cubilin in intracellular compartments, thereby eliminating apical membrane localization [21–23, 39]. The proteins are mutually dependent, which is also evident in IGS patients with the disease-causing mutation present in cubilin and in cubilin-deficient mice, where AMN is retained intracellularly [31, 40]. In vitro the cubilin–AMN complex is endocytically active driven by the intracellular endocytic motifs in the tail of AMN [22, 41]. Despite the possible expression of megalin in the intestine [12] AMN appears to facilitate the internalization of IF–B12 bound to cubilin in the intestine as evidenced by the absence of megaloblastic anaemia in megalin-deficient patients. In the kidneys, however, efficient uptake of filtered proteins requires a high-capacity endocytic system and in this setting CUBAM is unable to reabsorb filtered cubilin ligands in the absence of megalin as evidenced both in the megalin knockout mice and in megalin-deficient patients [31, 32]. These observations indicate that the molecular mechanisms for intestinal absorption of intrinsic factor–B12 are different from the proximal tubular reabsorption of protein. This does not exclude that AMN is able to mediate endocytosis of cubilin independently of megalin in the proximal tubule, as observed when IF–B12 is injected in megalin-deficient mice; however, the uptake is significantly reduced [31].

Cubilin function

Serving as an endocytic receptor cubilin mediates the uptake of proteins and protein-bound substances both in the intestine and in the kidneys (Figure 3). Intrinsic factor, synthesized by the gastric parietal cells and complexed to B12 in the duodenum, is the sole, known physiological ligand for cubilin, in the terminal ileum. In the kidneys proximal tubule cubilin mediates the uptake of several filtered proteins [42] and the combined action of cubilin and megalin essentially clears the ultrafiltrate of filtered proteins, lipids, vitamins and hormones resulting in an almost protein-free final urine [28]. Albumin was among the first ligands to be identified showing the importance of cubilin for urinary albumin excretion [43, 44]. It is now evident from the complete lack of endocytic uptake of albumin in cubilin-deficient mice that cubilin with the assistance of megalin is the only physiologically important albumin receptor in the proximal tubule [31, 45] (Figure 4). Additional ligands include vitamin carrier proteins, lipoproteins, other carriers, immune- and stress-related proteins and drugs [42, 46] (Table 1).

The identification of these ligands indicated the molecular basis of the low-molecular-weight (LMW) proteinuria often observed in IGS patients [5, 6]. Proteinuric IGS patients were recognized to hold mutations abolishing membrane localization of cubilin, whereas patients with mutations affecting only the intrinsic factor–B12-binding site do not present with LMW proteinuria (unpublished results) as originally demonstrated for vitamin D-binding protein (VDBP) [47]. Cubilin ligands may be divided into ligands that bind only to cubilin and ligands that bind to both megalin and cubilin (Table 1). Most ligands have been identified by in vitro-binding studies, and the identification has been established by urinary excretion studies in mouse [31, 45] and dog models [43, 47–49] as well as in patients suffering from IGS [40].
Cubilin in bowel and kidneys

Fig. 4. Immunofluorescent demonstration of endogenous albumin uptake in cubilin mosaic knockout mouse kidneys. Albumin (red) is seen only in cells expressing cubilin (green).

Table 1. Ligands for cubilin

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrinsinc-factor vitamin B_{12}</td>
<td>Nykjaer et al. 2001 [47]</td>
</tr>
<tr>
<td>Vitamin D-binding protein*</td>
<td>Birn et al. 2000 [43]</td>
</tr>
<tr>
<td>Other carrier proteins</td>
<td>Gburek et al. 2002 [83]</td>
</tr>
<tr>
<td>Albumin*</td>
<td>Gburek et al. 2003 [82]</td>
</tr>
<tr>
<td>Myoglobin*</td>
<td>Kozyraki et al. 2001 [49]</td>
</tr>
<tr>
<td>Haemoglobin*</td>
<td>Kozyraki et al. 1999 [48]</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Kozyraki &amp; Gburek 1999 [48]</td>
</tr>
<tr>
<td>Lipoproteins</td>
<td>Kozyraki &amp; Gburek 2003 [82]</td>
</tr>
<tr>
<td>Apolipoprotein AI</td>
<td>Kozyraki et al. 2000 [43]</td>
</tr>
<tr>
<td>High-density lipoprotein</td>
<td>Kozyraki &amp; Gburek 2002 [83]</td>
</tr>
<tr>
<td>Enzyme and enzyme inhibitors</td>
<td>Kozyraki &amp; Gburek 2003 [82]</td>
</tr>
<tr>
<td>Recombinant activated factor VIIa*</td>
<td>Seested et al. 2011 [88]</td>
</tr>
<tr>
<td>Immune- and stress-related proteins</td>
<td>Storm et al. 2011 [40]</td>
</tr>
<tr>
<td>Immunoglobulin light chains</td>
<td>Batuman et al. 1998 [89]</td>
</tr>
<tr>
<td>Clara cell secretory protein</td>
<td>Burmeister et al. 2001 [90]</td>
</tr>
<tr>
<td>α1-microglobulin*</td>
<td>Storm et al. 2011 [40]</td>
</tr>
<tr>
<td>Drugs and toxins</td>
<td>Tauris et al. 2009 [36]</td>
</tr>
<tr>
<td>Aminoglycosides*</td>
<td>Batuman et al. 1998 [89]</td>
</tr>
<tr>
<td>Receptors</td>
<td>Moestrup 1998 [13]</td>
</tr>
<tr>
<td>Megalin</td>
<td>Fyne et al. 2004 [22]</td>
</tr>
<tr>
<td>AMN</td>
<td>Fyne et al. 2004 [22]</td>
</tr>
<tr>
<td>Others</td>
<td>Fyne et al. 2004 [22]</td>
</tr>
</tbody>
</table>

*Designate ligands shared with megalin.

Notable, cubilin [47], as well as megalin [50], bind and mediate the proximal tubule uptake of vitamin D in complex with VDBP which is essential for the final, renal hydroxylation and activation of the vitamin [50]. The role of cubilin in the renal handling of ligands is comparable in mouse and man, although small differences have been observed. In proteinuric patients with IGS, increased urinary excretion of albumin, Apo AI and VDBP is observed [40], whereas this is not the case in mice revealing increased urinary excretion of albumin only [31]. Furthermore, some endocytosis of VDBP is observed in the absence of megalin in humans [32], which is not the case in mice [31, 50]. The role of cubilin in fetal development apparently also differs in mice and man as it has been reported that the deletion of cubilin in mice is incompatible with survival [51], whereas the receptor is anticipated to be less important or at least complemented by other factors in humans as evident from the survival of cubilin-deficient patients [40].

Cubilin function and dysfunction in disease

As may be anticipated from the expression pattern and functional studies of cubilin, receptor dysfunction has been implicated in vitamin B_{12} deficiency due to defective intestinal absorption and in renal dysfunction with tubular proteinuria.

Imerslund-Gräsbeck syndrome

Imerslund-Gräsbeck syndrome (IGS, hereditary megaloblastic anaemia, OMIM #261100) is usually diagnosed on the basis of failure to thrive, recurrent infections, and fatigue accompanied by megaloblastic anaemia developing in early childhood. Once treated the prognosis of the disease is favourable with stable proteinuria and kidney function [52], although, as mentioned the proteinuria is not corrected by B_{12} supplementation.

Several different mutations in CUBN and AMN have been identified as the cause of IGS [53]. Mutations in CUBN dominate in Finnish patients, mutations in AMN dominate in Norwegian cases, while both have been identified in the Middle East [54, 55]. The common functional implication of these different mutations is insufficient ability to mediate intestinal absorption of IF–B_{12}. This may be caused by (i) a single amino acid substitution within the IF–B_{12}-binding site of the CUBN [15] or (ii) mutations or gene deletions likely to result in absence of expression of functional cubilin [15, 40, 56], which also includes mutations in AMN [21, 22]. The proteinuria observed in some patients is due to the defective tubular re-absorption of filtered proteins including albumin [40]. Increased urinary excretion of the cubilin ligand VDBP was identified in a Finnish patient with a mutation resulting in early truncation of the receptor, while this was not observed in a patient with a single amino acid substitution in the IF–B_{12}-binding site [47]. This suggests that the proteinuria is related to specific mutations affecting the function of cubilin other than IF–B_{12} binding, which would also include mutations in AMN (unpublished results). Although this relationship has not been consistently described [55, 57], albuminuria with no reported B_{12} deficiency has recently been associated with a single-base, homozygous deletion in CUBN located differently from the known IF–B_{12} binding site [58], supporting the notion that the proteinuria may be dependent on the type of mutation. Varying degrees of proteinuria have been reported in IGS with protein/creatinine ratios ranging from a few hundred mg/g up to ∼2 g/g [40, 57] or comparable to 24-h protein excretions [56, 59, 60]. The urinary protein excretion is correlated with the excretion of...
cubilin ligands, such as albumin, α1-microglobulin and transferrin, whereas the correlation between the total protein excretion and urinary β2-microglobulin, a ligand for megalin, and immunoglobulin-G was less obvious [57]. So far no consistent changes in the renal structure have been reported in biopsies from cases of IGS. In one patient, with concomitant haematuria, focal mesangial expansion with immunoglobulin-A deposits consistent with coexisting IgA nephropathy was observed [40], while in others no or non-specific glomerular changes have been described [60–62]. Although cubilin has been identified in the podocyte [34], no characteristic glomerular pathology has been reported in animal models of cubilin deficiency or dysfunction, and thus the specificity of the glomerular changes observed in a few IGS patients remains elusive, and may in fact represent bias in the selection of patients for renal biopsy. In conclusion, current evidence suggest that proteinuria observed in patients with IGS is the result of tubular dysfunction; however, further studies in humans are needed. The tubular origin of the proteinuria in IGS is supported by the observation that generally the proteinuria is stable and not associated with progressive loss of renal function [52].

Urinary tract abnormalities have been reported in Norwegian patients with IGS [5]; however, as these are associated with mutations in AMN this may be caused by other developmental functions of AMN and independent of cubilin. Similarly, impaired transport of B12 into the CNS was suggested in one patient with IGS associated with a mutation in AMN [63], and despite the expression of cubilin in other tissues than intestine and kidneys, no consistent phenotype relating to these tissues have been reported in IGS.

Cubilin, albuminuria, and chronic kidney disease

Albuminuria is one of the most sensitive and widely used markers of kidney dysfunction and an important risk marker for the progression of renal disease as well as for mortality and death [64]. The importance of cubilin in tubular albumin uptake suggests a crucial role in regulating urinary albumin excretion. As discussed, mutations in cubilin and AMN often result in albuminuria; however, it is not clear whether cubilin dysfunction is associated with the progression of renal disease or with the increased mortality associated with albuminuria in larger populations. A genome-wide association study has identified, a single-nucleotide polymorphism (SNP) in CUBN as a gene locus for albuminuria both in the general population and in diabetes [65], supporting a role of cubilin in regulating urinary albumin excretion. Whether this link is casual and whether this is associated with a greater risk of progressive renal disease remain to be established. As also mentioned above, a single base pair deletion of CUBN exon 53 (~CUB domain 20) was recently found by exome sequencing in two siblings with albuminuria but no megaloblastic anaemia [58]. Recently, a different SNP in cubilin was associated with a greater risk for end-stage renal disease, as well as proteinuria and graft failure in transplanted kidneys, with the latter being associated with the donor rather than the recipient genotype [66].

While any causal relationship is uncertain, this may suggest that cubilin function or dysfunction may play a role for the development or progression of CKD although, as evident from patients with IGS, this is not obligatory.

Changes in cubilin function and/or expression have been reported in animal models of acute and chronic renal disease including lipopolysaccharide-induced endotoxemia [67], ischaemia–reperfusion kidney injury [67, 68], and chronic kidney disease using the remnant kidney model of chronic kidney disease [69]. Increased urinary excretion of cubilin, along with megalin, has been identified in models of Alport syndrome [70], and experimental as well as human diabetes [71, 72]. In cystic fibrosis mouse models [73], an increased excretion of cubilin but not of megalin was observed together with increased LMW proteinuria of cubilin ligands. Whether these changes are secondary or part of the primary events leading to kidney dysfunction is not clear. Excessive tubular uptake of filtered proteins, including albumin, has been implicated in the progression of chronic kidney disease [74] as also recently reviewed [75, 76]. Studies have suggested a number of mechanisms 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 [74, 76, 77]. Antisense RNA delivered by adenovirus and leading to partial knockdown cubilin is shown to inhibit adriamycin-induced glomerulosclerosis and tubulointerstitial damage in rats despite higher level of albuminuria [78]; however, additional studies are needed to establish the role of cubilin-mediated albumin uptake for the progression of proteinuric kidney disease [76].

Cubilin in acute kidney injury

Cubilin has been shown to bind and mediate uptake of several nephrotoxic proteins including light chains [79–81], myoglobin [82] and haemoglobin [83], which may cause acute kidney failure if filtered in excessive amount. Since all these proteins also bind to megalin, the specific role of cubilin in mediating nephrotoxicity is unresolved.

Other diseases

In Dent’s disease, proximal tubular expression of cubilin is significantly reduced [84] together with a decreased expression of megalin [84, 85] probably as a consequence of a disturbed intracellular trafficking of the receptors, leading to LMW proteinuria. Association studies have identified polymorphisms in CUBN associated with type 1 diabetes [86] and spina bifida [87], observations which need confirmation.

Conclusion

The physiological importance of cubilin in both the intestine and the kidneys is evident both from animal models and humans. The interaction of cubilin with other membrane proteins is also well established; however, further studies are needed to identify the tissue specificity of
these interactions and to clarify the significance of these in the individual, intracellular transport steps. The functional role of cubilin expression in podocytes awaits further clarification. Finally, while the phenotype of rare, inherited diseases involving mutations and dysfunction of cubilin or associated proteins is well described, the possible role of cubilin in the development of albuminuria and CKD as suggested by gene association studies requires further confirmation and clarification of the underlying pathogenic mechanisms.

Acknowledgements. The work was supported in part by the Danish Medical Research Council, Lundbeck Foundation, Novo Nordisk Foundation, Aarhus University and the program of the European Community, EUNEFRON (FP7, GA#201590).

Conflict of interest statement. None declared.

References

1. Minot GR, Murphy WP. Treatment of pernicious anemia by a special diet. J Am Med Assoc 1926; 87: 470–476
41. Pedersen GA, Chakraborty S, Steinhauser AL et al. AMN directs endocytosis of the intrinsic factor-vitamin B12 receptor cubam by engaging ARH or Dab2. Traffic 2010; 11: 706–720
71. Tschallik KN, Nimmo T, Bunn RC et al. Microalbuminuria in type 1 diabetes is associated with enhanced excretion of the endocytotic multiligand receptors megalin and cubilin. Diabetes Care 2009; 32: 1266–1268


88. Seested T, Appa RS, Jacobsen C et al. Recombinant activated factor VII is reabsorbed in renal proximal tubules and is a ligand to megalin and cubilin. *Nephron Exp Nephrol* 2011; 117: e82–e92


Received for publication: 11.9.12; Accepted in revised form: 29.10.12