Identification and Characterization of Two Distinct Ligand Binding Regions of Cubilin*

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Using polymerase chain reaction-amplified fragments of cubilin, an endocytic receptor of molecular mass 460 kDa, we have identified two distinct ligand binding regions. Region 1 of molecular mass 71 kDa, which included the 113-residue N terminus along with the eight epidermal growth factor (EGF)-like repeats and CUB domains 1 and 2, and region 2 of molecular mass 37 kDa consisting of CUB domains 6–8 bound both intrinsic factor-cobalamin (vitamin B₁₂; Cbl) (IF-Cbl) and albumin. Within these two regions, the binding of both ligands was confined to a 110–115-residue stretch that encompassed either the 113-residue N terminus or CUB domain 7 and 8. Ca²⁺ dependence of ligand binding or the ability of cubilin antiserum to inhibit ligand binding to the 113-residue N terminus was 60–65%. However, a combination of CUB domains 7 and 8 or 6–8 was needed to demonstrate significant Ca²⁺ dependence or inhibition of ligand binding by cubilin antiserum. Antiserum to EGF inhibited albumin but not IF-Cbl binding to the N-terminal cubilin fragment that included the eight EGF-like repeats. While the presence of excess albumin had no effect on binding to IF-Cbl, IF-Cbl in excess was able to inhibit albumin binding to both regions of cubilin. Reductive alkylation of the 113-residue N terminus or CUB 6–8, CUB 7, or CUB 8 domain resulted in the abolishment of ligand binding. These results indicate that (a) cubilin contains two distinct regions that bind both IF-Cbl and albumin and that (b) binding of both IF-Cbl and albumin to each of these regions can be distinguished and is regulated by the nonassisted formation of local disulfide bonds.

The gastrointestinal uptake of cobalamin (vitamin B₁₂; Cbl) occurs bound to gastric intrinsic factor (IF) by receptor-mediated endocytosis via a cell surface receptor, intrinsic factor-cobalamin receptor (IFCR) (1). IFCR isolated from canine ileal mucosa was estimated to have a molecular mass around 220 kDa and bound IF-Cbl with high affinity (2), and further proteolysis of this receptor revealed that IF-Cbl binding occurred with a number of receptor fragments of molecular masses as low as 80 kDa (3). In addition to the intestinal ileal mucosa, very high levels of IFCR were also detected in mammalian kidney (4) and in rat yolk sac (5). In contrast to canine ileal mucosal IFCR, the purified rat renal IFCR demonstrated a molecular mass of 457 kDa based on its amino acid and amino sugar content. It bound 2 mol of IF-Cbl (4) and like the yolk sac IFCR (5) was developmentally regulated (6). Although the physiological significance for the high levels of IFCR expression in the kidney was not known for a number of years, using proximal tubular polarized epithelial opossum kidney cells it was demonstrated that the apically expressed IFCR was able to internalize IF-Cbl and mediate Cbl transport from the apical to basolateral medium (7). A later study using a canine model with selective inherited intestinal Cbl malabsorption syndrome (8) demonstrated that in these animals the apical brush border membrane levels of IFCR in both ileal mucosa and kidney were depleted suggesting that the IFCR expressed in both tissues was a product of the same gene.

Although it was thought for the last decade that the renal IFCR may have some other function, its structure was only recently delineated (9), and a number of subsequent studies have shown that the renal IFCR is indeed a multifunctional receptor that is able to bind a variety of protein ligands with differing affinities. These include IF-Cbl (1–2 nM) (4), albumin (0.63 μM) (10), high density lipoprotein (0.1 and 56 nM) (11), and α and γ light chains (160 and 12 μM) (12, 13). The presence of low and high affinity binding sites for high density lipoprotein and light chain suggested that there are at least two binding sites for these ligands.

Based on the recent elucidation of its sequence, IFCR is now renamed cubilin mainly because it consists of a contiguous stretch of 27 CUB domains that represent nearly 88% of its total mass of 460 kDa and because IFCR binding has been shown to be localized to a region from CUB domain 5 to CUB domain 8 (14). A CUB domain is a 110–115-amino acid module present in developmentally regulated proteins that is known to form a β-barrel. The acronym CUB is derived from proteins complement Clr/Cls, Uegf, and bone morphogenic protein-1 that have these domains. Upstream of these CUB domains, the rest of the cubilin molecule contains a 113-residue N-terminal region followed by eight EGF-like repeats. Consistent with these structural observations, the new name of cubilin will be used instead of IFCR throughout the rest of the manuscript.

Despite these studies many aspects of the structure-function relationship of cubilin are poorly understood, particularly its ability to function as a multifunctional receptor, and the current studies were directed in addressing some of these issues. The results of the current studies show that cubilin contains two distinct ligand binding regions, one the 113-residue N terminus and the other CUB domains 7 and 8 that bind both IF-Cbl and albumin. Binding of both these ligands to each of these regions can be distinguished and is regulated by the
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8-EGF like domains
N 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

27-CUB domains

113 residue
N+EGF

+8 EGF +
CUB 1 & 2
CUB 5 CUB 6 CUB 7 CUB 8
CUB 9-12 CUB 12-17 CUB 18-27 CUB 7-8
CUB 6-8

Fig. 1. Schematic representation of various fragments of rat cubilin generated by reverse transcriptase PCR. Other details are provided under “Experimental Procedures.”

formation of local disulfide bond(s) that form spontaneously in the absence of molecular chaperones and exogenously added oxidized glutathione.

EXPERIMENTAL PROCEDURES

Materials—Sepharose and rat serum albumin were purchased from Sigma-Aldrich. pSec Tag B vector was from Invitrogen (Carlsbad, CA), and canine pancreatic microsomes and the TNT quick coupled transcription/translation system were from Promega (Madison, WI). Fluoro-Hance™ used for autoradiography was obtained from Research Products International Corp. (Mount Prospect, IL). Rat gastric intrinsic factor used in the current studies was purified from the rat stomach as described previously (15). Antiserum to purified rat renal cubilin raised in rabbits was prepared as described previously (4). Polyclonal antiserum to human EGF raised in rabbits was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Carrier-free Na125I for iodination and canine pancreatic microsomes and the TNT quick coupled translation system from Promega. The 35S-translated products were then used for in vitro cotranslational processing and post-translational modification of 35S-Express protein labeling mix from Fluka, and radioactivity was determined using the rat renal membranes (25,000 to 50,000 dpm) were incubated with 500 μl of a 1:1 suspension of Sepharose beads in 10 mM Tris-HCl, pH 7.4, containing 10 mM CaCl2 or 10 mM EDTA. In some experiments, the labeled cubilin fragments were preincubated with 2–5 μl of polyclonal antiserum to either cubilin or EGF. For experiments shown in Figs. 7 and 8 the addition of 5 μl of the polyclonal antiserum to cubilin and EGF resulted in maximum inhibition of ligand binding, and the addition of a higher amount (10–25 μl) of the antiserum did not inhibit ligand binding further. Using similar amounts (50,000 dpm) of protein synthesized from constructs CUB 7, CUB 8, or CUB 6–8, 5 μl of antiserum was sufficient to precipitate >90% of all three labeled proteins. Competition of ligand binding was carried out similarly except that the labeled cubilin fragments were preincubated for 60 min at 22 °C with either rat IF-Cbl (50 ng) or rat serum albumin (100 ng) prior to their binding to Sepharose linked to albumin or rat IF-Cbl, respectively. Following binding for 60 min, the beads were exhaustively washed with 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl (15–20 ml), and radioactivity bound was then released by boiling the beads with SDS sample buffer and subjected to nonreducing SDS-PAGE.

SDS-PAGE and Fluorography—35S-Labeled translation products or the ligand affinity-purified cubilin fragments were subjected to nonreducing SDS-PAGE (12%). Gels were fixed and then treated with Fluoro-Hance™ for about 30 min (as described by the manufacturer), and the bands were visualized by fluorography. In some experiments, the labeled cubilin fragments were first reduced with 2-mercaptoethanol (2%) and then alkylated with N-ethylmaleimide (1 mM) prior to SDS-PAGE. The bands were quantified by the AMBIS radioimaging system. SDS-PAGE data shown in Figs. 2–4 and Figs. 11 and 12 are typical representations of at least three separate translation and ligand binding experiments and reductive alkylation experiments, respectively.

Other Methods—Rat renal apical membranes were isolated by the Ca2+ aggregation method as described previously (4). The Ca2+-dependent binding of ligand, IF-[125I]Cbl (100–2500 pg), or 125I-rat serum albumin (10–200 ng) (specific activity, 200,000 dpm/μg of albumin) was determined using the rat renal membranes (25–50 μg of protein). The total ligand bound in the presence of 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM CaCl2 was subtracted from that bound in the presence of the same buffer but containing 10 mM EDTA to obtain Ca2+-specific ligand binding. Sequence alignment of the N-terminal region and CUB domains 7 and 8 were performed by Jellyfish version 1.4 using matrix-Gonnet.
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RESULTS

In Vitro Transcription/Translation and Ligand Binding of Reverse Transcriptase PCR-generated Cubilin Fragments—The various regions of cubilin that were amplified by reverse transcriptase PCR are shown in Fig. 1. Initially all cubilin fragments were in vitro transcribed and translated to test for their ability to synthesize a functional protein. Earlier studies (14) had revealed that when the media collected from stably transfected cells were tested for IF-Cbl binding, only the media from cells transfected with CUB domains 5–8 bound IF-Cbl, and none of the other cubilin fragments demonstrated IF-Cbl binding activity. In our studies (Fig. 2), four cubilin fragments encompassing CUB domains 1–4, 9–12, 12–17, and 18–27 were efficiently translated in vitro (lanes 1) and produced major proteins of molecular masses 45, 47, 70, and 100 kDa, respectively, consistent with the number of CUB domains present in each fragment. Each CUB domain consists of 110–115 residues with an average polypeptide mass of around 110 kDa. The [35S]methionine-labeled proteins synthesized by these cubilin fragments failed to bind either IF-Cbl (lanes 2) or albumin (lanes 3). In contrast, the ~71-kDa cubilin fragment synthesized (Fig. 3A, lane 1) from the N-terminal fraction, which contained the 113-residue N-terminal region along with the eight EGF-like repeats and CUB 1-2 demonstrated Ca2+-dependent binding of both IF-Cbl (Fig. 3B, lane 1) and albumin (Fig. 3C, lane 1). Within this region, the 113-residue N-terminal region, which synthesized a protein of ~18 kDa (Fig. 3A, lane 2) was by itself enough and sufficient to bind both IF-Cbl and albumin (Fig. 3, B and C, lanes 2). The cell-free translation of the 113-residue N terminus resulted in the synthesis of a predominant 18-kDa band, but there were three other bands of higher molecular mass (36, 39, and 44 kDa). These higher molecular mass forms may represent the aggregated forms of the 18-kDa form, and the size difference between them could be due to different amounts of Triton X-100 bound to them. This conclusion is based on the observation that all three forms bound both ligands. Prior incubation of the translated product with Sepharose alone did not eliminate these bands from the fraction that was eluted from ligand affinity matrix (data not shown). The DNA fragment encoding the eight EGF-like repeat region synthesized a protein of 45 kDa but did not bind either of the two ligands (data not shown). Since our earlier data (Fig. 2) had demonstrated that no ligand binding activity occurred with cubilin fragments synthesized downstream of CUB domain 9 and CUB domains 1–4, attention was focused to test ligand binding with cubilin fragments synthesized by CUB domains 5–8. While all of the individual CUB domains between 5 and 8 were able to synthesize protein products of molecular masses between 17 and 20 kDa (Fig. 4A), only the proteins synthesized with CUB 7 and CUB 8 were able to bind both IF-Cbl (Fig. 4B) and albumin (Fig. 4C). In addition, protein products of molecular mass 28 or 37 kDa synthesized from CUB 7-8 or 6–8, respectively, also bound both the ligands (Fig. 4, B and C). Lack of ligand binding (Fig. 4, B and C) observed by protein synthesized by CUB 5 or CUB 6 was not due to low levels of protein expressed by these constructs since the amount of [35S] radioactivity used for affinity ligand binding was 3–4 times higher than that used for SDS-PAGE (Fig. 4A) analysis of the translated products. The two ligand binding regions of cubilin, the 113-residue N terminus, which included the eight EGF-like repeats along with cubilin domains 1 and 2, and CUB domains 6–8 region contained three and five potential N-glycosylation sites, respectively. Thus, we wanted to test whether these sites are utilized for N-glycosylation and if so, whether glycosylation at these site(s) affects ligand binding. Thus, these constructs were translated in vitro in the presence and absence of canine pancreatic microsomes. SDS-PAGE analysis (Fig. 5) failed to detect any shift in the electrophoretic mobility (Fig. 5A) with either region 1 representing the N terminus + eight EGF-like repeats + CUB domains 1 and 2 (Fig. 5A, compare mobility in lanes 3 and 4) or region 2 containing CUB domains 6–8 (Fig. 5A, compare mobility in lanes 1 and 2). Moreover, both regions of cubilin bound the ligands whether they were synthesized in the absence (Fig. 5, B and C, lanes 1) or the presence (Fig. 5, B and C, lanes 2) of pancreatic microsomes. Taken together, these observations suggested that under the experimental conditions used in the present studies these sites are not N-glycosylated.

![Fig. 2. SDS-PAGE analysis of 35S-labeled CUB domain fragments.](image)

![Fig. 3. SDS-PAGE analysis of 35S-labeled N-terminal cubilin fragments.](image)

![Fig. 4. SDS-PAGE analysis of 35S-labeled CUB domain (5–8) fragments.](image)
and that the ligand binding is not affected when cell-free translation was carried out with the cotranslational addition of canine pancreatic microsomes. However, a positive control incubation to test the activity of the pancreatic microsomes indicated that under similar experimental conditions, *Saccharomyces cerevisiae* α-factor was processed with core N-glycosylation (Fig. 5A, compare mobilities of lanes 5 and 6) and that *Escherichia coli* β-lactamase is processed with cleavage of its signal peptide (Fig. 5A, lanes 7 and 8). These observations indicated that the lack of core N-glycosylation of the cubilin fragments was not due to the use of an inactive sample of canine pancreatic microsomes.

**Ca**

Dependence of Ligand Binding—The binding of IF-Cbl to its receptor is **Ca**

-dependent, but it is not known whether the dependence on **Ca**

for ligand binding is a property of the full-length receptor or can be demonstrated with functionally active regions of cubilin. To address this issue, the binding of **Ca**

-labeled cubilin fragments were tested for **Ca**

dependence of binding to both IF-Cbl and albumin (Fig. 6). The binding of IF-Cbl and albumin to native renal brush border membrane was inhibited by EDTA by 97 and 50%, respectively. EDTA-inhibitable binding of IF-Cbl and albumin to region 1 encompassing only the 113-residue N-terminal region of cubilin was 64 and 70%, respectively. Interestingly, the inclusion of eight EGF-like repeats with the 113-residue N terminus resulted in binding of IF-Cbl and albumin to native renal brush border membrane. The bands visualized by fluorography were quantified using the AMBIS radioligand system. The dotted horizontal line represents the total ligand binding in the presence of 10 mM **Ca**

obtained using the indicated cubilin fractions or the native membranes (50 μg of rat renal apical brush border membrane protein) and is taken to represent 100%. The ratio of image density obtained for binding (**Ca**

/EDTA) is expressed as percentage of EDTA-inhibitable binding. The values obtained are mean ± S.D. from triplicate binding assays performed using four different translation experiments.

On the other hand, the binding of albumin to CUB 7 and CUB 8 was inhibited between 55 and 60% and reached 95% for CUB 6–8 (Fig. 7). Studies with antisera to EGF (Fig. 8), which is relevant to only region 1, revealed inhibition of albumin binding by nearly 90%, while it had very little or no effect on IF-Cbl binding to this region.

**IF-Cbl Inhibits Albumin Binding at Both Regions of Cubilin**—To examine whether the two protein ligands compete for binding, binding of one ligand was carried out after a preincubation of the **Ca**

-labeled cubilin fragments with the other ligand. Preincubation with excess albumin did not inhibit the binding of IF-Cbl by either the 113-residue or the CUB 6–8 fragment (Fig. 9). On the other hand, when similar experiments were carried out with preincubation in the presence of IF-Cbl, the binding of albumin by both regions of cubilin was inhibited by >90–96%.

**Local Disulfide Bond Formations in the Functional Regions of Cubilin Are Important for Ligand Binding**—Sequence analysis of the 113-residue N-terminal ligand binding region of cubilin re-
Cubilin is a 460-kDa multidomain, multifunctional endocytic receptor expressed in the apical membranes of tissue epithelial cells and functions synergistically (16) with megalin, another endocytic receptor of molecular mass 660 kDa. For an endocytic receptor, cubilin is unique in that it has no discernable transmembrane domain (9), and it is not fully understood how cubilin interacts with the apical lipid bilayer membrane to expose its ligand binding sites to allow binding of water-soluble ligands such as IF-Cbl and albumin.

The identification of two ligand binding regions localized to the first one-third of the cubilin molecule (Fig. 2–4) suggested strongly that in the context of full-length cubilin both these regions must be outside the lipid bilayer so that they are able to bind the water-soluble ligands. One of the regions of cubilin identified in this study, the 113-residue N terminus, binds both IF-Cbl and albumin (Fig. 3). The N terminus of cubilin has also been demonstrated to form coiled-coil α-helices (17) that are amphipathic in nature (14) and are thought to interact with the lipid bilayer. The amphipathic nature of the 113-residue N terminus is evident as it formed aggregates following its synthesis (Fig. 3), and all the aggregated protein also bound ligand. Moreover, when this region along with the EGF-like repeats was stably expressed in Chinese hamster ovary cells, it was retained within the cell unlike other fragments of cubilin, which were all secreted efficiently. However, the ligand binding ability of this fraction was not studied (14). Thus, based on the ability of this fragment to bind the ligands and its hydrophobic nature, the N-terminal region of cubilin may interact with the outer leaflet of the apical bilayer membrane. Recently it has been shown (18) that the cubilin fragment containing both the N terminus and the EGF-like repeats along with CUB domains 1 and 2 bind to megalin in a Ca$^{2+}$-dependent manner. Moreover, this fragment when reconstituted into egg phosphatidylcholine vesicles containing 10 mol % cholesterol in the presence of megalin demonstrated decreased (75%) binding to these lipid vesicles. Taken together, these observations strongly suggest that megalin binding to the N-terminal region may displace this region to the outer leaflet of the bilayer to allow its ligand binding site to be exposed.

In addition to the N terminus, there is evidence that the entire cubilin molecule is peripherally localized to the apical lipid bilayer at multiple sites with a variety of membrane interactions that could include protein-lipid, protein-protein, and divalent cation-dependent. With this type of topography, it is very likely that both ligand binding regions are exposed for ligand binding. These conclusions are based on a number of observations obtained from different studies. These observations include (a) partial solubilization of canine intestinal cubilin bound to either native brush border or synthetic lipid vesicles by phospholipases, detergents, and proteases (19), (b) partitioning of intestinal cubilin in the detergent-poor aqueous phase following treatment of apical brush border membrane with Triton X-114 (20), (c) partial solubilization of native renal membrane-bound cubilin with a number of reagents that included EDTA, heparin, and phosphatidylethanolamine (9, 17), (d) full-length purified renal cubilin of molecular mass 467 kDa bound to Triton X-100 micelles bound 2 mol of IF-Cbl/mol (4) in a Ca$^{2+}$-dependent manner, and (e) the functional topography of either native brush border- or lipid vesicle-bound cubilin is the same in that the ligand binding and antigenic sites of cubilin are exposed outside the lipid bilayer (19).

Another important consideration of these studies is the observation that the two functional regions of cubilin identified in this study are able to bind the ligand when translated in vitro in the presence or absence of pancreatic microsomes (Fig. 5, B...
and C). Thus, it is unlikely that the potential N-glycosylation sites present in these regions are utilized for N-glycosylation, and the lack of core N-glycosylation may not influence localized folding of the functional regions of cubilin to affect ligand binding. Although estimating the number of N-linked sugars based on electrophoretic mobility shift in proteins with larger molecular mass is not accurate, earlier studies of rat (6) and opossum (21) renal cubilin have shown that cubilin is indeed N-glycosylated, and a more recent estimate of the number of N-linked sugars suggests that rat renal cubilin may be N-glycosylated at 28–30 of 42 potential sites (9). Thus, it is possible that the 12–14 potential N-glycosylation sites that are not utilized may also include the potential sites present in the two ligand binding regions of cubilin. Alternatively, in these shorter cubilin fragments, unlike in the full-length cubilin, the potential N-glycosylation sites may not be accessible for N-glycosylation, particularly considering that the cubilin constructs used did not contain the N-terminal signal sequence that is essential for the recognition of the protein synthesized by the endoplasmic reticulum membranes.

Although the two distinct ligand binding regions of cubilin are confined to 110–113 residue units, their Ca$^{2+}$ dependence for ligand binding appear to be influenced by the flanking regions. This is particularly so in region 2 (Fig. 6) where the presence of CUB domain 6 increased Ca$^{2+}$ dependence but not...
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the total IF-Cbl binding of the CUB 7-8 fragment. However, the inclusion of CUB domain 6 did not further enhance the Ca\(^{2+}\)-dependent binding of albumin by this cubilin fragment. In addition, the Ca\(^{2+}\)-dependent binding of both IF-Cbl and albumin rose dramatically when both CUB domains 7 and 8 were present together rather than being present as individual domains. These observations suggested that there may be multiple Ca\(^{2+}\) binding sites localized both near and away from the ligand binding sites in CUB domains 7 and 8. The location, number, and nature of the Ca\(^{2+}\) interaction may influence whether the Ca\(^{2+}\) requirement is absolute or obligatory, and further studies are needed to identify the Ca\(^{2+}\) binding sites of the cubilin fragments. One region of cubilin that may contain potential Ca\(^{2+}\) binding sites are the EGF-like repeats as these structures, present in many proteins (22), have been implicated in Ca\(^{2+}\) binding. While both IF-Cbl and albumin binding to this region demonstrated 60–70% Ca\(^{2+}\) dependence, inclusion of all the EGF-like repeats with the 113-residue N terminus actually eliminated Ca\(^{2+}\) dependence of albumin but not IF-Cbl binding (data not shown). Thus, Ca\(^{2+}\) dependence of ligand binding to cubilin could be regulated by the location of the Ca\(^{2+}\) binding loop structure that is formed, and it is likely that the position of these loops is different depending on the ligand of choice.

In many ways the two functional regions of cubilin identified in this study share many of the same properties as the full-length protein. These include Ca\(^{2+}\)-dependent ligand binding (Fig. 6), the ability of cubilin antiserum to inhibit the binding of both ligands (Fig. 7), and the ability of IF-Cbl to inhibit albumin binding but not the ability of albumin to inhibit IF-Cbl binding (Fig. 9). It is interesting to note that there is a around 750–750 higher affinity for IF-Cbl binding relative to albumin binding by the purified full-length cubilin, and this appears to be true at both functional regions of cubilin as well (Fig. 9). Ligand binding to the N-terminal region that also included the EGF repeats could be distinguished as the EGF antiserum was able to inhibit binding of albumin by >90%, while it had no effect on IF-Cbl binding (Fig. 8). Thus, it is likely that within each ligand binding region of cubilin, the albumin and IF-Cbl binding sites are spatially related, yet they can be distinguished. However, additional studies are needed to fully understand the spatial relationship of multiple ligands binding to these functional units of cubilin and also how its nonfunctional units influence the Ca\(^{2+}\) dependence of ligand binding.

Sequence comparison (Fig. 10) of the ligand binding regions of cubilin revealed only 7% identity between the N-terminal region and CUB domains 7 and 8. While the N terminus had between 12–14% identity with either CUB domain 7 or 8, the two CUB domains by themselves had close to 30% identity. CUB domain 8 contained a proline residue at the 85th position (corresponding to codon 1297 in the full-length cubilin) that has been implicated in the high affinity binding of IF-Cbl (23). Mutation of this proline to leucine resulted in a 5-fold decrease of affinity for IF-Cbl. Moreover, the mutation, P1297L appears to be specific and is present in some (Finnish) but not other (Norwegian and Saudi Arabian) patients with hereditary megaloblastic anemia (24). It is interesting to note that a proline residue is also present at a similar position in CUB domain 7 and in N terminus. In addition, a proline residue is also conserved at the 85th position (Fig. 10) in all three ligand binding regions. It is not known whether the proline residue at the 95th position in the N terminus or CUB domain 7 or the proline conserved in all three regions at position 85 has any role in ligand binding. In a canine model of inherited Cbl malabsorption syndrome (8), the cubilin defect was due to its misfolding that resulted in the failure of cubilin delivery from the endoplasmic reticulum to the apical membranes. It is obvious that much needs to be learned about the molecular defects of cubilin that cause defective uptake of IF-Cbl in the intestine or of albumin in the proximal tubular cells that result in the development of Cbl deficiency and proteinuria, respectively. Although proteinuria is often a common finding in patients with Cbl malabsorption syndrome (25), some patients do not develop proteinuria (26, 27). These studies indicate that different mutations of the cubilin molecule may exist that affect uptake of IF-Cbl, albumin, or both.

The formation of a disulfide bond and its importance in ligand binding is evident in both ligand binding regions of cubilin. In the 113-residue N terminus, reductive alkylation resulted in the formation of an extended form with lower electrophoretic mobility on SDS-PAGE (Fig. 11). This bond is formed between Cys-63 and Cys-117 as these are the only two cysteine residues present in this ligand binding region (Fig. 10). Alternatively, in the second ligand binding region there is also evidence of disulfide binding within CUB domains 6–8, formation of which is essential for ligand binding (Fig. 11B). Within this region, intra-CUB domain disulfide bonding appears to be important as both CUB 7 and CUB 8 appear to form disulfide bonds (Fig. 12) that are important for ligand binding (data not shown). It is interesting to note that the functional cubilin fragments synthesized are able to form disulfide bonds in the absence of cotranslationally added pancreatic microsomes or oxidized glutathione. Addition of both these components cotranslationally has been implicated in the formation of disulfide bonds and cotranslational translocation of newly synthesized proteins (28, 29) such as the 46-kDa mannose 6-phosphate receptor (30). The spontaneous formation of functionally correct disulfide bonds in the absence of added pancreatic microsomes suggested that the folding of the mature cubilin may be hierarchal proceeding from secondary structure via subdomains and domains toward the complete tertiary structure. Local folding required for the acquisition of ligand binding of cubilin fragments may be rapid, not needing the assistance of molecular chaperones such as protein disulfide isomerase, which is known to assist in protein folding and disulfide bond formation (31). In addition, the spontaneous formation of correct disulfide bonds in cubilin may aid in the early acquisition of ligand binding as well as its rapid vectorial delivery to the plasma membrane. Previously studies (32) have shown that cubilin expressed in opossum kidney cells is very rapidly transported to the plasma membrane with a t\(_{1/2}\) of 30 min.

In summary, we have demonstrated that cubilin, a multifunctional receptor, is able to bind both albumin and IF-Cbl at two distinct regions. Within each region the binding of these two ligands occurs at spatially related sites and is dependent on disulfide bonds formed within each of these regions.

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