Cubilin expression and posttranslational modification in the canine gastrointestinal tract

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Cubilin expression and posttranslational modification in the canine gastrointestinal tract. Am J Physiol Gastrointest Liver Physiol 279: G748–G756, 2000.—Cubilin is an endocytic receptor of the apical brush border membrane that is essential for intrinsic factor-mediated cobalamin absorption in small intestine. However, cubilin is more highly expressed in kidney and yolk sac, and recent molecular characterization of the receptor has focused on these tissues. The aim of this investigation was to examine tissue-specific cubilin expression and post-translational modifications with an emphasis on the gastrointestinal tract. Intrinsic factor-cobalamin binding activity, cubilin immunoreactivity, and cubilin mRNA levels were determined in multiple segments of canine gastrointestinal mucosa and other tissues. These aspects of cubilin expression varied in parallel, suggesting that the major determinant of regional cubilin expression in the gastrointestinal tract is modulation of cubilin mRNA. Cell fractionation indicated that ileal cubilin is not strongly membrane associated. An ~185-kDa brush border specific and two >400-kDa precursor forms of cubilin were identified. Asparagine-linked oligosaccharide modifications characterized by differential glycosidase digestion of affinity-purified cubilin from ileal mucosa and renal cortex differed, but ileal and renal intracellular cubilin comigrated on SDS-PAGE at ~400 kDa after oligosaccharide removal, thus reconciling previous conflicting size estimates of the cubilin polypeptide.

Cubilin cDNAs of 11.3–11.6 kb encoding a highly conserved 3,620-amino acid sequence were cloned, first from rat yolk sac and kidney (30) and subsequently from human (23) and dog (39) kidney. The encoded IF-cbl binding receptor, previously called IFCR, has been named cubilin in recognition of its unique structure, including a tandem arrangement of 27 CUB domains (initially found in complement components C1r/C1s, Uegf, and bone morphogenetic protein-1), and in recognition that it is a multiligand receptor. In addition to IF-cbl, recently identified cubilin ligands include apolipoprotein A-I and high-density lipoproteins (17, 22), albumin (6), and immunoglobulin light chains (5). Northern and Western blots of rat tissues demonstrated cubilin expression in kidney, small intestine, and yolk sac, but not in liver (30). Renal cubilin appears to be a peripheral membrane protein because it lacks a transmembrane domain or glycosphospholipid anchor addition signal in the deduced amino acid sequence. Consistent with this, purified canine intestinal cubilin behaved as a peripheral membrane protein when reconstituted in artificial liposomes (33). Published evidence suggests that an association with megalin (gp330), an apical plasma membrane receptor of the low-density lipoprotein receptor family, may mediate cubilin, and therefore IF-cbl, endocytosis, and subsequent cubilin recycling to the apical membrane (7, 30). The size of detergent-solubilized and affinity-purified canine intestinal cubilin has been determined variously as 180–230 kDa by gel filtration, SDS-PAGE, or amino acid analysis (32, 34). In contrast, the cDNA of canine renal cubilin suggests a molecular mass of at least 400 kDa (39). For this reason, some investigators have questioned the identity of intestinal and renal cubilin (15).

COBALAMIN (vitamin B<sub>12</sub>, cbl) is an essential micronutrient that all higher animals, including humans, must obtain from dietary sources (20). In the proximal gastrointestinal tract, cbl binds to intrinsic factor (IF), a 50-kDa glycoprotein produced by gastric mucosa in many species but mainly by pancreas in dogs and cats (4, 11, 37). Endocytosis of the IF-cbl complex is mediated by a specific receptor expressed in intermicrovillar clefts of apical brush border membranes on villus tip enterocytes of the distal small intestine (18, 26). Binding of cbl by IF and brush border expression of the receptor on ileal enterocytes are required for gastrointestinal cbl absorption.

In addition to ileum, IF-cbl binding activity and immunoreactive receptor protein are expressed in re-
Intestinal cbl absorption is limited by cubilin expression in various normal and disease states, but the molecular nature of cubilin regulation is unknown. As an initial step in the investigation of regulation of intestinal cubilin expression, we have examined regional expression of cubilin ligand binding activity, immunoreactivity, and mRNA along the longitudinal axis of canine gastrointestinal tract and demonstrate that constitutive cubilin expression in intestine is determined by mechanisms controlling cubilin mRNA levels. Cell fractionation studies indicate that canine intestinal cubilin behaves as a peripheral membrane protein and that apparent differences in the size of intestinal and renal cubilin are due to tissue-specific posttranslational modifications.

**MATERIALS AND METHODS**

Reagents. The following were purchased from commercial sources: $^{57}$Co-radiolabeled cyanocobalamin (300 μCi/nmol) from Amersham International (Little Chalfont, UK); anti-rabbit IgG-agarose, anti-rabbit IgG-alkaline phosphatase conjugate, p-nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate from Sigma Immunochemicals (St. Louis, MO); disodium 2-chloro-5-(4-methoxy-3,2'-5'-chloorotricyclo[3.3.1.1^5.7^]decan)-4-yl)-1-phenyl phosphate (CDP-Star) from Roche Molecular Biochemicals (Indianapolis, IN); TRIzol reagent from Life Technologies/GIBCO BRL (Gaithersburg, MD); and endoglycosidase H (endo H) and peptide N-glycosidase F (PNGase F) from New England Biolabs (Beverly, MA). Rat stomach IF was prepared and radiolabeled by formation of IF-$^{35}$Co complexes as described (35).

Animals and tissue isolation. Animal husbandry was provided by Michigan State University Laboratory Animal Resources, and animal-use protocols were approved by the All-University Committee for Animal Use and Care. Dogs weighing 12–20 kg were fasted overnight. Surgical anesthesia was induced by intravenous injection of thiopental sodium (15 mg/kg) and maintained by isoflurane inhalation. Five-centimeter segments of small intestine were resected at sequential 30-cm intervals beginning 10 cm distal to the pyloroduodenal junction in some dogs and 10 cm proximal to the ileocecal junction in others. A 5-cm segment of descending colon and 25 cm$^2$ of gastric fundus were also resected. The mucosa of each segment was scraped from the underlying muscularis and snap frozen in liquid nitrogen. In the distal small intestine of some dogs, samples of mucosa overlying Peyer’s patches were scraped and frozen separately from the adjacent absorptive mucosa. Other tissues were removed simultaneously with interruption of the blood supply of each and were snap frozen. After surgery, each dog was euthanized, without recovery from anesthesia, by intravenous overdose of pentobarbital sodium (85 mg/kg body wt).

Ligand binding activity determination. Resected samples of gastrointestinal mucosa were thawed and homogenized on ice in 9 volumes of cold 20 mM Tris·HCl, pH 7.5, containing (in mM) 150 NaCl (TBS), 5 KCl, protease inhibitor cocktail [2 each of benzamidine and N-ethyl maleimide, 1 phenylmethylsulfonyl fluoride (PMSF)], and 7.5 NaN$_3$. Total protein concentrations were determined by the method of Lowry (28). Ca$^{2+}$-specific receptor binding of a saturating amount (370 fmol) of IF-$^{35}$Co was determined in aliquots of mucosal homogenates containing 1.25–2.5 mg of total protein by a rapid microcentrifuge method essentially as described (16). Under these conditions, binding was never $>95$ fmol (0.25 of added ligand), and both free and bound ligand were measured after the incubation period.

Immunoprecipitation and Western blot analysis. Cubilin from canine renal cortex was used to produce anticybilin antisera for two reasons: 1) cubilin is far more abundant in renal cortex than in ileal mucosa (14) and 2) to examine the cross-immunoreactivity of canine ileal and renal cubilin. Canine renal cubilin was affinity purified to homogeneity as previously described (14), and rabbits were immunized by subcutaneous injection of the protein in oil/water emulsion. Rabbit serum was harvested, and anticybilin activity was characterized. Ten microliters of the polyclonal serum blocked 86% of IF-cbl binding to 700 fmol of the purified renal cubilin preparation used for immunizations. The antiserum detected a single major band on Western blots of renal cortex homogenates (see Fig. 4).

Resected intestinal mucosal samples were thawed and homogenized on ice in 4 volumes of cold 50 mM Na-phosphate buffer, pH 7.4, containing 100 mM NaCl, 20 mM EDTA, and protease inhibitor cocktail. Triton X-100 and heparin were added to the homogenates to 2% and 250 U/ml, respectively. They were incubated at 4°C overnight with constant agitation and then centrifuged at 20,000 g for 40 min. Aliquots of the supernatants containing 6 mg of total protein were incubated for 4 h at 4°C after sequential additions of 20 μl preimmune rabbit serum and 30 μl anti-rabbit IgG agarose beads and were centrifuged at 12,000 g for 2 min to remove nonspecific binding proteins. Cubilin was immunoprecipitated from the precleared supernatants by sequential addition of 20 μl anti-cubilin rabbit serum and 30 μl anti-rabbit IgG agarose beads, again with 4 h incubations at 4°C after addition of each reagent. The protein-agarose bead complexes were washed three times in TBS containing 0.4% Na-deoxycholate and 1 mM PMSF and boiled in 20 μl nonreducing SDS-PAGE loading buffer. Solubilized proteins were separated by SDS-PAGE on 5% gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBS with 0.2% Triton X-100 (TBST) and 2% gelatin at 22°C for 4 h and incubated at 4°C overnight in TBST with 0.4% BSA and anti-cubilin rabbit serum (1:20,000). After copious washing in TBST, membranes were incubated at 4°C for 3 h in TBST with 0.4% BSA and anti-rabbit IgG-alkaline phosphatase conjugate (1:30,000). After copious washing in TBST, immunoreactive proteins were detected a single major band on Western blots of renal cortex homogenates (see Fig. 4).

Northern blot analysis. Frozen tissue samples were homogenized in TRIzol reagent, and RNA was isolated according to the manufacturer’s protocol. Thirty micrograms of total RNA from each sample was electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon membranes by standard protocols (31). A loading control probe of canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was produced by RT-PCR of canine testicular RNA as previously described (13). Similarly, an ~15-kb marker was produced by hybridizing blots to a random prime-labeled probe of partial canine megalin (gp330) cDNA produced by RT-PCR of canine kidney cortex RNA. The probe was amplified using PCR primers 5'-GGCTTCTACATCTGAATGTGTC-3' and 5'-CACAGACTTGTGTGGTCTACCC-3', corresponding to sequences at nucleotide positions 9618–9640 and 10157–10178 of the human megalin cDNA sequence, respectively (GenBank accession no. U38387). The PCR product was confirmed to be megalin cDNA by sequencing and demonstrated 88%
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identity with the corresponding portion of the human megalin cDNA. Blots were hybridized to random prime-labeled partial canine cubilin cDNA, extending between positions 3922 and 4907 of the sequence (GenBank accession no. AF137068), marker, and/or control probes and washed by standard methods (31). Hybridizing transcripts were detected by exposing blots to autoradiographic film or to a storage phosphor screen. The relative intensity of probe hybridization between samples was determined quantitatively from the storage phosphor screen with the Imagquant program (Molecular Dynamics, Sunnyvale, CA).

Tissue fractionation, purification of IF-cbl binding proteins, and oligosaccharide analysis. Frozen ileal mucosa was thawed and homogenized in cold TBS with protease inhibitors, and an aliquot was centrifuged at 40,000 g for 1 h. The resulting supernatant was again centrifuged at 150,000 g for 2 h, and IF-cbl binding proteins were affinity purified from each fraction and Western blotted as described below. In separate experiments, frozen canine ileal mucosa or renal cortex was thawed in 9 volumes of cold 2 mM Tris-HCl, pH 7.4, containing 50 mM mannitol and 2 mM benzamidine (TMB) and homogenized in an iced blender on high in four 15-s bursts. An aliquot was centrifuged at 4°C at 40,000 g for 1 h. The pellet comprising total membranes was resuspended in TBS with 1 mM PMSF and 2 mM benzamidine (TBSP). The supernatant was made 10 mM CaCl₂ by addition of concentrated solutions of each. The remaining 10% TMB homogenate of cold TMB and homogenized again. The homogenate was stirred, incubated on ice for 10 min, and centrifuged at 2,500 g for 15 min. The membrane pellet was homogenized in 9 volumes of cold TBSP, and the supernatant was centrifuged at 16,000 g for 40 min. The resulting brush border-enriched pellet was homogenized with 10 strokes in a precooled Potter-Elvehjem in 4 volumes of cold TBS with 2 mM benzamidine. The final supernatant was made 10 mM Tris-HCl, 140 mM NaCl, and 1 mM PMSF by addition of concentrated solutions of each. Total protein and alkaline phosphatase activity of each fraction were determined as described previously (14).

Each of the above fractions was made 1% in Triton X-100 and agitated continuously at 4°C overnight. Each was centrifuged at 40,000 g for 1 h, and the supernatants were made 5 mM CaCl₂. IF-cbl binding proteins were purified from each fraction by affinity chromatography on individual 0.4-ml columns of rat gastric IF-cbl-agarose bead matrix, essentially as described for purification of canine renal cubilin (14). The proteins were eluted from the affinity matrix in TBS, pH 5.0, containing 5 mM EDTA and 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. CaCl₂ and Tris base were added to each eluted fraction to 5 mM and pH 7.4, respectively, and the eluted proteins were dialyzed against 1 mM benzamidine at 4°C overnight. Aliquots of the dialyzed proteins were reduced in volume by vacuum centrifugation, separated on 5% SDS-PAGE gels, and Western blotted as above, except that immunoreactive proteins were detected by incubation of the blot with a chemiluminescent alkaline phosphatase substrate (CDP-Star) and exposure to autoradiographic film. Control blots included both substitution of the primary antibody with nonimmune rabbit serum and elimination of the primary antibody altogether. Analysis of asparagine-linked (N-linked) oligosaccharides was by endo H or PNGase F digestion of the dialyzed proteins as previously described (14), electrophoretic separation by SDS-PAGE, and Western blotting. The only change from the manufacturer’s protocol was that samples were denatured by boiling in 0.5% SDS without 2-mercaptoethanol before glycosidase digestion. Data analysis. For comparison of intestinal tracts of different lengths, the position of each small intestine mucosal sampling site was expressed as the percentage that the length of gut from pyloroduodenal junction to sampling site was of the entire small intestinal length measured between the pyloroduodenal and ileocecal junctions. Because phosphorimager analysis of Northern blots was not in physiologically relevant units, quantitation of cubilin probe hybridization to RNA from different mucosal samples was normalized to equal GAPDH hybridization values and expressed as a fraction of the maximum hybridization determined in each dog intestine. To compare specific IF-cbl binding activity and cubilin mRNA expression, the IF-cbl binding data from each sample site were expressed similarly in Fig. 3. Small intestinal distributions of cubilin ligand binding and mRNA between the most proximal nonzero values and the maximal values were examined by linear regression analysis. Densitometry of autoradiographic film exposed within the linear response range was performed on a Gel Doc 2000 with Quantity One quantitation software from Bio-Rad (Hercules, CA).

RESULTS

Gastrointestinal cubilin expression. Tissue specificity of cubilin mRNA expression was evaluated in multiple canine tissues by Northern blot hybridization (Fig. 1) by using a 1-kb portion of the canine cubilin cDNA cloned from renal proximal tubule cells as probe (39). Specific hybridization of the cubilin probe was detected in ileum but not in any other portion of the gastrointestinal mucosa, salivary gland, or liver. Cubilin probe hybridization was strong in kidney cortex but was nearly undetectable in kidney medulla. This pattern of expression was consistent with findings in rat tissues (30), although the canine yolk sac equivalent was not examined. In ileum and kidney cortex a strongly hybridizing transcript of 11–12 kb and a larger but more faintly hybridizing transcript were observed consistently. On overexposed blots of ileal RNA, one or two even larger transcripts were sometimes observed.

![Fig. 1. Tissue specificity of cubilin mRNA expression. Thirty micrograms of total RNA from each of several canine tissues were electrophoresed and blotted. Membranes were hybridized simultaneously to cubilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Equality of loading was determined by examination of the 28S ribosomal RNA on the ethidium bromide-stained gel. The ~15-kb position was determined from subsequent hybridization to a canine megalin (gp330) cDNA probe.](http://apd.physiology.org/)

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**Stoma**
**Duodenum**
**Ileum**
**Colon**
**Saliy gland**
**Esophagus**
**Liver**
**Spleen**
**Kid. cortex**
**Kid. medulla**
**Brain**
**Lung**

Cubilin: ~15kb, ~4.5kb
GAPDH: 28S
Sequential portions of gastrointestinal mucosa were examined for expression of cubilin ligand binding activity, immunoreactivity, and mRNA expression. Specific activity of EDTA-inhibited IF-cbl binding in multiple segments of gastrointestinal mucosa, from stomach to colon, of five dogs is shown in Fig. 2A. In each dog, IF-cbl binding activity was detected in the distal one-half of small intestine, with highest specific activity in the mucosa at 90% of the distance from pyloroduodenal to ileocolic junctions, corresponding to 30–40 cm proximal to the ileocolic junction. Ligand binding activity was reduced to 0.5–0.7 of the peak value in the most distal segment of small intestine in each dog. In four other dogs, IF-cbl binding activity of mucosa overlying distal small intestinal Peyer’s patches (24 ± 6.6 fmol/mg protein) was compared with that of the adjacent absorptive mucosa (47 ± 1.4 fmol/mg protein). Thus reduced ligand binding activity in the most distal ileal segment was probably due to modification of the epithelium overlying the nearly-circumferential Peyer’s patch observed in the last 10–15 cm of canine small intestine. Cubilin ligand binding activity was not measurable in stomach or colonic mucosa.

Western blots of proteins immunoprecipitated with anti-renal cubilin antiserum from multiple segments of gastrointestinal mucosa from each dog demonstrated immunoreactive cubilin only in the fractions exhibiting ligand binding activity and demonstrated a pattern of cubilin immunoreactivity that paralleled the observed pattern of ligand binding (Fig. 2B). Four immunoreactive proteins were observed on nonreducing gels in mucosa of distal small intestinal segments, but none was observed in proximal small intestinal, stomach, or colonic mucosa. There was a band just under 200 kDa and two bands at >400 kDa. The band observed at high apparent relative molecular mass ($M_r$) was also observed in canine renal cortex (Fig. 4). None of these four cubilin species was observed when the immunoprecipitation or immunodetection on Western blots was performed with nonimmune serum (not shown). Thus small intestinal expression of immunospecific cubilin corresponded to expression of cubilin ligand binding activity.

Cubilin mRNA expression in multiple segments of gastrointestinal mucosa was examined in each dog by Northern blot (Fig. 2C). As demonstrated in Fig. 1, no hybridizing CUBN transcripts were observed in stomach or colonic mucosa. Hybridization of the cubilin probe to mucosal RNA was observed only in samples isolated from the distal half of small intestine, and highest expression was found in mucosa at 85–95% of the distance from pyloroduodenal to ileocolic junctions. In all samples in which hybridization of the probe was observed, a strongly hybridizing transcript at 11–12 kb and a more faintly hybridizing transcript at 13–14 kb were seen, just as in the multiple tissue blot.

To compare the small intestinal distributions of the cubilin IF-cbl binding function and mRNA in a quantitative way, both sets of data for each dog were expressed as fractions of the peak activity observed (Fig. 3). In the proximal-to-distal direction, IF-cbl binding and cubilin mRNA in each dog were first detectable in mucosa at 45–50%, and both parameters were maximal at 85–90% of the small intestinal length. Regression analysis of the combined data of all five dogs in the interval from first detectable to maximal indicated that both parameters increased linearly (slopes of 2.1 and 2.0, respectively) with comparable variance (correlation coefficient values of 0.82 and 0.78, respectively). Thus, in this group of dogs, cubilin function and mRNA expression in the small intestine were highly correlated, both in distribution of detectable expression and the position of maximal expression.

Ileal cubilin membrane association. To determine whether endogenous cubilin in ileal mucosa behaves as a weakly bound peripheral membrane protein in vitro, as has been reported for renal cubilin (30), we examined the distribution of cubilin after homogenization and fractionation of ileal mucosa without detergent. Ileal mucosa was homogenized in neutral hypotonic buffer, and an aliquot was centrifuged at 40,000 g, separating soluble and total membrane fractions. Apical microvillus brush border membranes were enriched from the remaining homogenate by CaCl$_2$ aggregation.
of membranes followed by differential centrifugation, with recovery of brush border membranes in the 16,000 g pellet (21). In a typical fractionation of 1 g of ileal mucosa, IF-cbl binding activity was 3.1 and 3.9 pmol (0.10 and 0.18 pmol/mg protein) in the soluble and total membrane fractions, respectively, and 0.59 pmol (0.41 pmol/mg) in the brush border-enriched fraction, indicating 2.7-fold enrichment of cubilin ligand binding over the homogenate and recovery of 8.4% in the brush border fraction. This suggested that some portion of cubilin may have been redistributed from the membrane to soluble fractions.

To examine this more closely, IF-cbl binding proteins in each of the 40,000 g soluble, total membrane, and brush border-enriched fractions were examined by Western blot. However, to concentrate the cubilin proteins to immunodetectable levels, after fractionation the proteins in each fraction were solubilized in Triton X-100 and subjected to affinity chromatography. Proteins eluted from the affinity matrix were separated by nondetergent fractions of ileal mucosa (lanes 1–3) or renal cortex homogenate (lane 4) and separated by nonreducing SDS-PAGE and detected on Western blots with polyclonal anti-canine renal cubilin serum (Fig. 4). Three major cubilin bands were found in each ileal fraction. Two bands migrated just above and below the position of kidney cubilin recovered in parallel with size estimates of 550 and 450 kDa, respectively, and a diffuse band migrated at ~185 kDa. There were also two faint bands of very high apparent Mr in each fraction. In two independent experiments, the proportion of total cubilin in each fraction represented by each size cubilin form was determined by densitometry of exposed films. The diffuse brush border band (BB form) comprised 30% of recovered cubilin proteins in the soluble fraction, 55% in the total membrane fraction, and 60% in the brush border-enriched fraction, indicating an enrichment in the brush border fraction of 1.5- to 2-fold relative to the total homogenate. In addition, the smaller intracellular form (IC form) in the brush border fraction was depleted ~4-fold relative to that in the soluble fraction and ~2-fold relative to the total membrane fraction. The larger IC form represented ~20% of cubilin in all three fractions. The 145-kDa band was judged to be nonspecific cross-reactivity because it was always present in the chemiluminescent detection system; it was the only band detected and with no decrease in signal on films of control blots developed with nonimmune rabbit serum as the primary antibody, with the anti-rabbit IgG-ALP conjugate only, or with [125I]protein A. Detection with the latter two control reagents suggested that the 145-kDa band represented cross-reacting, nonreduced canine IgG from lymphocytes in ileal mucosa. The seeming enrichment of the very high apparent Mr form was not a consistent finding and often occurred in the other fractions.

The same set of three immunospecific cubilin bands was also recovered from the final supernatant after pelleting the brush border fraction at 16,500 g, a fraction likely to contain microsomes. To examine whether
cubilin had redistributed to a truly soluble fraction, ileal mucosa was again homogenized without detergent, but in neutral isotonic buffer without CaCl₂, and centrifuged at 40,000 g for 1 h. The resulting supernatant was centrifuged again at 150,000 g for 2 h. IF-cbl binding proteins were affinity purified from each fraction and Western blotted as above. All three of the cubilin forms identified above were present in each of these fractions, and there was no proportional enrichment of the BB form or diminution of the smaller IC form apparent in any fraction (not shown). Immunoreactive cubilin was roughly equally distributed between the 150,000-g supernatant and membrane pellet fractions. Thus none of the identified ileal cubilin forms appeared to be tightly membrane bound.

The IF-cbl binding proteins recovered from canine kidney cortex homogenate in parallel to those from ileal mucosa are shown in Fig. 4. In kidney there was a single major immunoreactive band that migrated at ~460 kDa, a position intermediate to the ileal IC forms of cubilin. The same single major band was detected by Western blot of canine kidney homogenate without prior fractionation or affinity chromatography, indicating that the anticubilin serum used as primary antibody was immunospecific in whole tissue homogenates. Inclusion of disulfide bond-reducing agents in the gel-loading buffer in preliminary experiments resulted in an increased apparent Mᵦ, but 10- to 20-fold reduction in signal, of renal cubilin and complete failure to detect ileal cubilin bands (not shown).

Oligosaccharide analysis. To further characterize the apparent size and tissue-specific differences of cubilin species, proteins purified from the 40,000-g soluble fraction of ileal mucosa and renal cortex homogenates by IF-cbl affinity chromatography were subjected to differential glycosidase digestion before nonreducing electrophoretic separation and Western blotting (Fig. 5). Renal (lanes 1–3) and ileal cubilin (lanes 4–9) were digested with endo H (lanes 2, 5, and 8) or PNGase F (lanes 3, 6, and 9). Lanes 1, 4, and 7 were mock digests with no enzyme added. The renal and ileal proteins in lanes 1–6 were from normal dog tissues, and the ileal proteins in lanes 7–9 were from mucosa of a dog affected with inherited selective cbl malabsorption due to failure of brush border cubilin expression (12, 14). There was nonspecific protein degradation apparent in both mock and glycosidase digestions of renal cubilin that was not apparent in digestions of ileal cubilin. Endo H digestion of renal cubilin appeared to shift its migration slightly, whereas PNGase F digestion reduced the apparent size to ~400 kDa. Endo H digestion of ileal proteins reduced the apparent size of the ~450-kDa band to ~400 kDa but had no effect on gel migration of the other major ileal cubilin species. In contrast, PNGase F digestion of ileal cubilin reduced the apparent size of both the ~550- and ~450-kDa bands to ~400 kDa and the ~185-kDa BB form to 145 kDa. These data indicate that the N-linked oligosaccharide content of mature (endo H resistant) renal cubilin is ~15% of its total mass but that of ileal cubilin is ~30%. The BB form was missing from ileal cubilin species of dogs affected with inherited cbl malabsorption, but the remaining species exhibited similar shifts in size to normal dog ileal cubilin after both glycosidase digestions.

**DISCUSSION**

Functional expression of a plasma membrane receptor may be regulated at one or more of multiple biosynthetic steps. The magnitude of changes in intestinal cubilin mRNA expression from segment to segment demonstrated here accounted fully for the changes in cubilin ligand binding activity and immunodetectable protein also observed, indicating that the most important molecular mechanism regulating regional distribution of cubilin expression in the canine gastrointestinal tract is modulation of cubilin mRNA levels. The sharp decline of both cubilin mRNA expression and ligand binding activity in the most distal segment of small intestine was most probably due to sampling from areas of modified epithelium overlying a high density of Peyer’s patches. The codistribution of cubilin function, immunoreactive protein, and mRNA expression correlated well with previous in vivo localizations of IF-cbl binding and absorption in canine small intestine by oral radiolabeled cbl administration (2, 8, 10, 29) and is similar to that demonstrated by mucosal ligand binding measurements in humans (16). In the present study, however, we measured the IF-cbl binding function of cubilin in homogenates of whole mucosa and did not differentiate the activity of cell surface receptor from that of intracellular cubilin. We cannot comment, therefore, on whether endocytic functions mediated by cubilin on the enterocyte surface may be
regulated also by regulation of receptor translocation or recycling to the plasma membrane, as has been described for glucose transporters (19), or by other posttranslational mechanisms.

In examining cubilin mRNA expression by Northern blot with a canine renal cubilin cDNA probe, two principal hybridizing transcripts were detected in distal small intestinal mucosa and renal cortex. The 11- to 12-kb transcript corresponds in size to the length of cloned renal CUBN cDNA (39) and hybridized ~4-fold more strongly than did the larger transcript. Because the blotted samples included nuclear RNA, the multiple hybridizing transcripts could represent mature cubilin mRNA and incompletely processed forms of the primary CUBN transcript still retaining one or more introns. As previously reported, Southern blots of canine DNA digested with several restriction endonucleases indicated only a single hybridizing CUBN locus in the canine genome (39), thus eliminating the possibility that we observed hybridization to multiple closely related transcripts of different genes. An alternative explanation that has not been examined is that the CUBN transcript may be alternatively spliced or polyadenylated.

Cubilin proteins detected by immunoprecipitation and Western blot were expressed in parallel to cubilin function measured by IF-cbl binding activity and CUBN mRNA measured by Northern blots. The polyclonal antibody used in this study was raised against purified renal cubilin of a single apparent Mr, but specifically detected three major proteins on Western blots of ileal mucosal proteins. This indicated that the multiple immunoreactive forms of cubilin observed in ileal mucosa were not simply members of a protein complex obtained by coimmunoprecipitation or IF-cbl binding of one member of the complex. In preliminary experiments, it was determined that some form of receptor concentration, either immunoprecipitation as in Fig. 2B or affinity chromatography as in Figs. 4 and 5, was necessary for reliable immunodetection of ileal cubilin on Western blots. Failure to do so could explain why cubilin was not detected on Western blots of ileal mucosa from a pair of Imerslund-Gräsbeck patients purported to overexpress cubilin ligand binding activity and from which only small amounts of mucosa were available (9).

What we have designated IC forms in Figs. 2B and 4 appear to be intracellular cubilin precursors at different stages of maturation. The N-linked oligosaccharides of the smaller IC form, estimated to be 450 kDa, were cleaved by endo H, indicating that they are a high-mannose type and derived from cubilin that had not been transported from the rough endoplasmic reticulum (RER) to the cis-Golgi. Furthermore, the 450-kDa band was found largely in the 40,000 g soluble fraction after homogenization in TMB without detergent and was depleted from the brush border-enriched fraction, consistent with an association with RER-derived microsomes. Oligosaccharides of the larger IC form, estimated to be 550 kDa, were endo H resistant but PNGase F susceptible, indicating that they were complex or hybrid types that had been exposed to Golgi processing enzymes. The same was true of oligosaccharides on the 185-kDa form of ileal cubilin.

We concluded that the ~185-kDa band (BB form; Figs. 2B and 4) is a brush border form of cubilin on the basis of 1) its enrichment in fractions enriched for brush border membranes and 2) its absence from ileal mucosa of dogs exhibiting an inherited defect that blocks brush border cubilin expression. It is likely that the ~185-kDa form arises by proteolytic cleavage of the ~550-kDa form when exposed to proteases in the intestinal lumen. This idea is consistent with previous findings by gel exclusion chromatography that in vitro trypsin treatment converted high-molecular-weight canine ileal cubilin to IF-cbl binding forms of 150–180 kDa (34). Cleavage of apical membrane proteins by luminal proteases of the gastrointestinal tract has also been demonstrated in vivo, as for aminopeptidase (38). As seen in Fig. 4, there was no analogous brush border cleavage of renal cubilin. It was previously demonstrated that the cubilin missing from renal brush border membranes of cbl malabsorption dogs was the ~460-kDa form that, in normal dog renal cortex, is an endo H-resistant form (14).

Thus, similar to most glycoproteins destined for the plasma membrane, it appears that canine ileal cubilin is translated as a 400-kDa polypeptide and cotranslationally glycosylated in the RER to ~450-kDa. N-linked oligosaccharides are subsequently modified to produce a mature ~550-kDa glycoprotein after departure from the RER, and the mature form is cleaved to ~185 kDa on the brush border membrane after exposure to proteases in the intestinal lumen. These experiments do not determine whether the 185- or ~550-kDa form of cubilin (or both) is responsible for IF-cbl endocytosis because, even though the ~550-kDa form is produced intracellularly, both forms may coexist for some time on the external brush border. If the endocytic function of cubilin is inactivated by cleavage to 185 kDa, this luminal processing step is nevertheless a marker of cubilin exposure to the intestinal lumen.

As shown in Fig. 5, dogs affected with an inherited defect blocking brush border cubilin expression and lacking the 185-kDa BB form still exhibit both IC forms of cubilin in intestine, including the endo H-resistant ~550-kDa form. As was demonstrated previously (14), a portion of renal cubilin of the affected dogs was also resistant to deglycosylation by endo H. These data suggest that the inherited block to cubilin expression in enterocytes and proximal tubule cells operates after cubilin matures to the endo H-resistant ~550-kDa form and before exposure to the luminal side of the brush border. Genetic linkage evidence indicates that the disease locus in these dogs is not the cubilin locus (39), and therefore some other gene product must mediate cubilin-specific transport from the site of N-linked oligosaccharide maturation, usually the Golgi, to the luminal brush border surface. Although a study in rat yolk sac suggests that some newly synthesized cubilin arrives on the brush border with endo
H-sensitive oligosaccharides (3), it may be that these phenomena are tissue specific.

Enrichment and recovery of total cubilin ligand binding activity and immunoreactivity of the ~185-kDa form was considerably less than that of the tightly associated brush border membrane marker ALP, but it was also clear that a portion of brush border cubilin redistributed to other fractions during homogenization. This study confirmed that endogenous cubilin is only loosely membrane associated in canine ileum, similar to what was previously noted for endogenous cubilin in rabbit kidney (30) and canine ileal cubilin reconstituted in artificial liposomes (33). These in vitro behaviors are consistent with designation of cubilin as a peripheral membrane protein.

The very high apparent Mₐ bands observed on Western blots of ileal and renal cortex (Figs. 2B and 4) were most likely aggregates of cubilin that survived boiling in SDS. We have observed these also on silver-stained gels of affinity-purified ileal and renal cubilin, so they are not Western blotting artifacts. The aggregate forms in ileal mucosa had a higher apparent Mₐ than in renal cortex (Fig. 4), and they shifted migration on SDS-PAGE after glycosidase digestions (Fig. 5). These observations were consistent with more extensive N-linked glycosylation of the 550-kDa form of ileal cubilin than that of renal cubilin evident from the relative shifts in gel migration after PNGase F digestion (Fig. 5). Heavy glycosylation of ileal cubilin may be an adaptation conferring some protection from luminal proteases and may explain the greater stability of ileal vs. renal cubilin apparent during glycosidase digestions in vitro.

Physiochemical evidence of cubilin aggregation under various in vitro conditions has been reported previously (7, 24, 27, 32, 34). In a recent report, trimers of bovine renal cubilin of ~1,500 kDa were resistant to disruption in 6 M guanidine but were separated into 440-kDa monomers after disulfide bond reduction (27). The NH₂-terminal region conjectured to mediate trimer formation has also been identified as a region required for membrane association of cubilin fragments expressed in transfected cells (24). In the latter study, aggregate forms were apparent on nonreducing gels only in the constructs expressing the membrane binding NH₂-terminal sequence. This region of cubilin also has the only unpaired cysteine in the entire protein that would be available for dimer formation. The presence of aggregate forms is, however, an inconsistent observation, so it remains unclear whether cubilin multimer formation occurs in vivo or is merely an in vitro artifact.

Detection of the ~450-kDa form was evidence that ileal cubilin gains IF-cbl binding activity and immunoreactivity at a step in biosynthesis before modification of high-mannose oligosaccharides to endo H-resistant forms and before expression in a position on the brush border appropriate to its endocytic function. Previous data indicated that the same was true of canine renal cubilin (14). At the steady states of tissues examined in this report, renal cubilin was mostly endo H resistant, but a large proportion of ileal cubilin was endo H sensitive. However, this does not reflect a true tissue-specific difference of cubilin partitioning between the RER and post-RER compartments because renal cortex cells that express cubilin are largely a population of fully differentiated cells, whereas ileal enterocytes that express cubilin are a mixture of proliferating and immature crypt cells, partially differentiated midvillus cells, and fully differentiated villus tip cells. Thus, relative to renal cortex, homogenates of ileal mucosa are enriched in cubilin forms produced early in the biosynthetic pathway, and the mature forms are underrepresented.

PNGase F removal of N-linked oligosaccharides from both IC forms of ileal cubilin resulted in comigration on SDS-PAGE with deglycosylated renal cubilin at ~400 kDa, consistent with the polypeptide molecular mass deduced from the canine renal cubilin cDNA (39). The evident tissue-specific differences of N-linked oligosaccharides may have contributed to discrepancies in the literature about ileal cubilin protein size, leading to thought that intestinal and renal cubilin ligand binding activities might be due to expression of different but closely related genes (15). The present data indicated that ileal and renal cubilin in dogs have the same size translation product. Moreover, both a cDNA probe and polyclonal antibody derived from renal cubilin detected gastrointestinal cubilin in the same pattern as determined by IF-cbl binding activity and as previously reported for canine gastrointestinal binding and absorption of oral cbl (2, 8, 10, 29).

Conversely, antibody raised against purified canine ileal cubilin demonstrated cross-immunoreactivity with canine renal cubilin in a previous study (14). These findings aside, however, the most elegant and conclusive demonstration that ileal and renal cubilin functions are expressions of the same gene are recent reports of a null mutation in the cubilin gene of a human patient exhibiting both intestinal IF-cbl binding activity and as previously reported for canine gastrointestinal binding and absorption of oral cbl (2, 8, 10, 29). Conversely, antibody raised against purified canine ileal cubilin demonstrated cross-immunoreactivity with canine renal cubilin in the literature (14). The present data indicates that ileal and renal cubilin functions are expressions of the same gene are recent reports of a null mutation in the cubilin gene of a human patient exhibiting both intestinal IF-cbl binding activity and as previously reported for canine gastrointestinal binding and absorption of oral cbl (2, 8, 10, 29).

REFERENCES


