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By

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ABSTRACT

CLONING AND CHARACTERIZATION OF CANINE INTRINSIC FACTOR-COBALAMIN RECEPTOR / CUBILIN

By

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Cubilin is a high molecular weight multiligand receptor that mediates intestinal absorption of intrinsic factor-cobalamin and selective protein reabsorption in renal tubules. The genetic basis of selective intestinal cobalamin malabsorption with proteinuria was investigated in a canine model closely resembling human Imerslund-Gräsbeck syndrome caused by cubilin mutations. Canine cubilin cDNA of 11,282 bp was cloned and sequenced, including 73 bp of 5' and 349 bp of 3' untranslated sequence. The open reading frame of 10,860 bp corresponds to a deduced amino acid sequence of 3,620 residues and demonstrates an overall amino acid identity of 83% with human and 70% with rat cubilin. Further characterization of tissue-specific cubilin expression and post-translational modification with emphasis on the gastrointestinal tract was carried out. Intrinsic factor-cobalamin binding activity, cubilin immunoreactivity, and cubilin mRNA levels were determined in multiple segments of canine gastrointestinal mucosa and other tissues. The parallel aspects of cubilin expression suggested that the major determinant of regional cubilin expression in the gastrointestinal tract was 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 in ileum, but only an ~460 kDa form in renal cortex. Asparagine-linked oligosaccharide modifications characterized by differential glycosidase digestion of affinity purified cubilin from ileal mucosa and renal cortex. Ileal and renal intracellular cubilin comigrated on SDS-PAGE at ~400 kDa after oligosaccharide removal, suggesting that the size differences were caused by different glycosylation.

Intragenic markers were identified within introns for both cubilin and megalin genes in the canine family and used to test the hypothesis of genetic linkage of the disease to cubilin or megalin loci. Megalin, a transmembrane receptor interacting with cubilin *in vitro* and colocalizing with cubilin *in vivo*, is speculated to anchor cubilin onto apical membranes of ileal enterocytes. Linkage of either gene was rejected, indicating that the canine disorder resembling Imerslund-Gräsbeck syndrome is caused by defect of a gene product other than cubilin or megalin. These results imply that there may be locus heterogeneity among human kindreds with selective intestinal cobalamin malabsorption and proteinuria and that normal brush-border expression of cubilin requires the activity of an accessory protein other than megalin.

This dissertation is dedicated to:

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CHAPTER 1

LITERATURE REVIEW

Overview of normal cobalamin absorption

Cobalamin (Cbl) is a complex organometallic substance consisting of a corrin ring, a central cobalt atom, and various axial ligands. The basic structure, known as vitamin B_{12}, is synthesized exclusively by microorganisms. In non-ruminant animals, bacterial synthesis takes place only in the large bowel and the cecum, and from these sites absorption cannot take place. Therefore, absorption of vitamin B_{12} is entirely from dietary sources (Booth and Mollin, 1959), including fecal contamination or coprophagy. Cbl is an essential micronutrient required for the health and well being of all higher animals, including human. All of these animals are capable of converting the vitamin into the two required coenzyme forms, adenosyl-cobalamin (AdoCbl) and methylcobalamin (MeCbl), which are essential cofactors for methylmalonyl-CoA mutase and methionine synthetase, respectively, in intermediate metabolism.

Cobalamin absorption, which is a complex process comprised of sequential protein-binding events, begins with a digestive phase (Figure 1.1). The vitamin is bound predominantly by haptocorrin (HC, R protein) in gastric juice and becomes bound to intrinsic factor (IF) after the HC moiety is partially degraded by pancreatic proteases in the duodenum (Allen et al., 1978; Carmel et al., 1983). IF is a Cbl-specific-binding protein classically synthesized in man by the gastric parietal cells of the fundus and body of the stomach (Christensen et al., 1973; Levine et al., 1980). IF is synthesized by various cells and glands

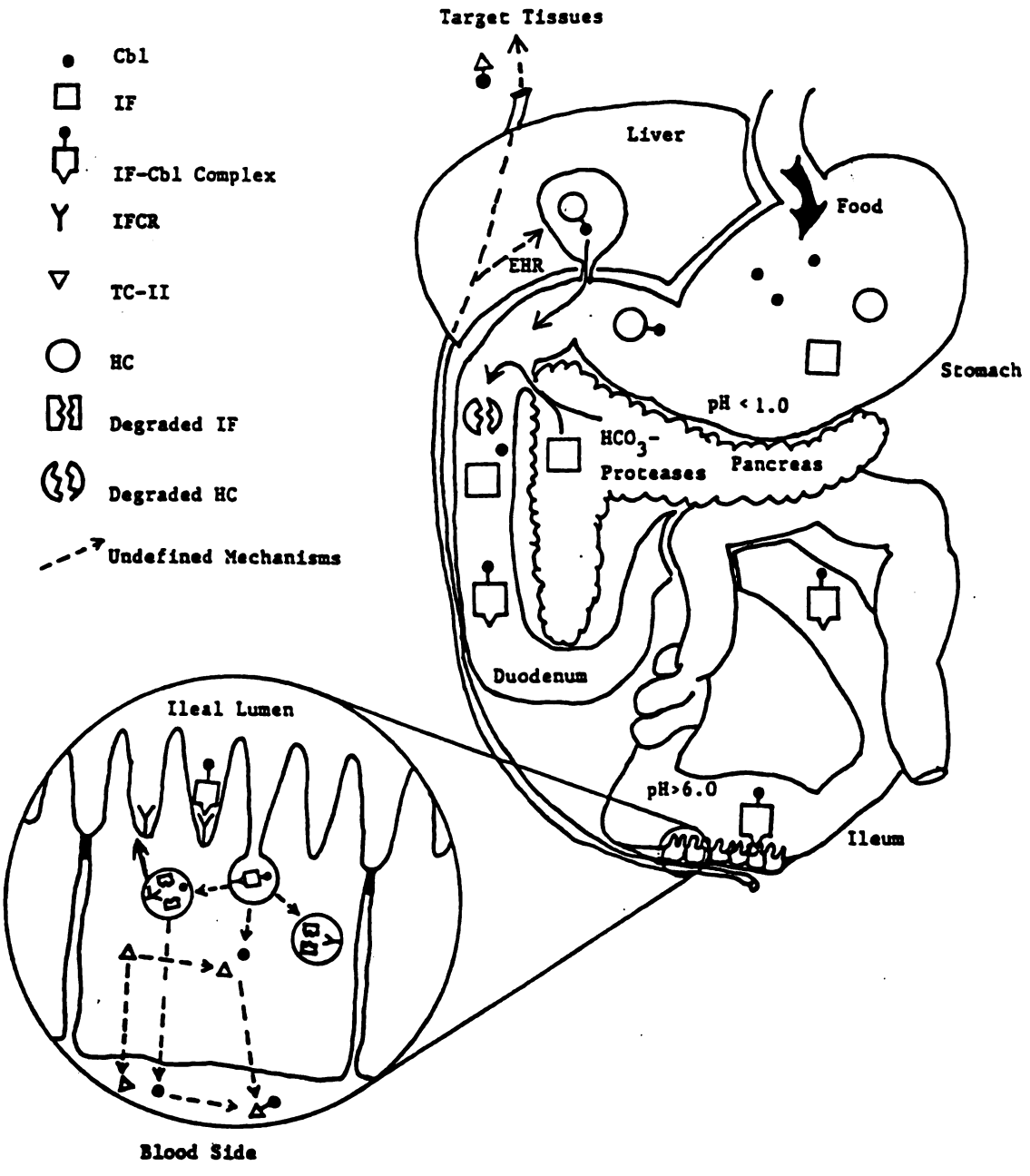

Figure 1.1 Canine intrinsic factor-mediated gastrointestinal cobalamin absorption.
Provided by Dr. John Fyfe.

derived from foregut depending on the different species (Gordon, 1989; Festen, 1991). IF is produced by the gastric mucosa of humans, pigs, and rats but is produced mainly in pancreatic duct cells in dogs (Batt and Horadagoda, 1989; Simpson et al., 1989; Vaillant et al., 1990; Simpson et al., 1993) and pancreas in cats (Fyfe, 1993). IF synthesized by different species showed different cross-species immunoreactivity (Hooper et al., 1973). Functional IF secretion and IF mRNA levels are developmentally regulated (Ramaswamy et al., 1989; Dieckgraete et al., 1988). IF secretion is also tissue specifically regulated, being activated by pentagastrin in the human stomach and by cholecystokinin in the canine pancreas after eating (Simpson et al., 1993), but the correlation between IF secretion and the expression of other Cbl-binding proteins is still unknown.

The IF-Cbl complex is highly resistant to proteolysis. This enables it to pass intact through the small bowel. Physiological cobalamin absorption takes place in the distal small intestine (Booth and Mollin, 1959), which has specific receptors for the IF-Cbl complex on the microvillous membranes of mucosal cells (Donaldson et al 1967; Levine et al., 1982). Attachment of the IF-Cbl complex to the receptor requires the presence of divalent cations (notably calcium) (Mackenzie et al., 1972) and a pH between 6 and 9 (Cooper and Castle, 1960; Herbert and Castle, 1961) but is independent of temperature and energy (Donaldson et al., 1967). Intrinsic factor-cobalamin receptor (IFCR), a membrane protein of the ileal brush border, mediates intestinal uptake and initiates transcytosis of dietary and biliary Cbl across the mucosal barrier. The number of receptors in the intestine seems to be very low. The greatest density of ileal

receptor sites is exhibited in dogs and pigs, being approximately 0.3 $x10^{12}$ to 4.9 x 10^{12} binding sites/ gram mucosa (Hooper et al., 1973). The restricted absorption of cobalamin is partly explained by the small number of receptors. IFCR is specific for IF-Cbl complex while free cobalamin, free IF and Cbl attached to other Cbl binding proteins cannot bind the receptor (Hooper et al., 1973; Katz and cooper, 1974). There is an enterohepatic cycle of Cbl absorption. The daily amount of Cbl excreted in bile constitutes approximately 0.1 to 0.19% of the total amount stored in the human body (Nicolas JP and Guéant JL, 1995). Pancreatic enzymes are responsible for the degradation of biliary R protein leading to the transfer of the released Cbl to biologically active IF and eventually to Cbl absorption in the terminal ileum (El Kholty et al., 1991).

Following the adsorption of the IF-Cbl complex to the ileal receptor there is a delay of 3 to 4 hours before the vitamin appears in the blood (Doscherholmen et al., 1959; Fyfe et al., 1991a). The processes involved in the translocation of cobalamin from the ileal receptor to the blood are still largely unknown, though some knowledge has accumulated. The receptor mediated endocytosis of IF-Cbl complex occurred within 30 min after the complex attached to its ileal surface receptor (Kapadia et al., 1983). *In vivo* and *in vitro* studies with ^{57}Co-labeled cyanocobalamin and [^{35}S]methionine-labeled IF showed that following endocytosis, the IF-cobalamin complex was released from the receptor and transferred to the lysosomal or prelysosomal acidic vesicles, where IF was degraded with a half-life of 4 hours (Kapadia et al., 1983; Ramanujam et al., 1991a, Guéant et al., 1992; Dan et al., 1994; Birn et al.,1997). Chloroquine or

cycloheximide treatment reduced the transport of Cbl suggesting release of Cbl from IF required acid pH, that there was a series of compartments involved in the transport of Cbl, and that new protein synthesis was required (Robertson JA., 1985a; Robertson JA, 1985b; Ramaswamy et al., 1989) The receptor of IF-Cbl complex is rapidly recycled onto the brush-border membrane after endocytosis (Dan and Cutler, 1994; Le Panse et al., 1997), and the liberated Cbl exported from lysosomes via a specific transporter (that malfunctions in Cbl F) to transcobalamin II (TC II). The location for this transport is still an open question. Some evidence suggested that Cbl released from IF and transfered to TC II within the rat enterocytes (Robertson and Gallagher, 1985b; Ramaswamy et al., 1989; Ramanujam et al., 1991a). But other researchers questioned if the crude homogenate used for TC II-[^{57}Co]Cbl immunoprecipitation can provide sufficient discrimination between the tissue components of the ileum (Quadros et al., 1999). TC II is a specific Cbl transport protein which has been detected in many cell types in culture (Hall et al., 1985) and in human and rat tissues (Li et al., 1994). In dogs, all the plasma Cbl is bound to TC-II (Rappazzo and Hall, 1972). In contrast, in human, only newly absorbed Cbl is bound to TC-II, and most circulating Cbl is bound to TC-I which is haptocorrin (HC) (Gullberg R., 1972). This distinction is important since, in dogs, TC-II deficiency would be expected to have extremely low serum Cbl concentration, but in human TC-II deficiency, serum Cbl concentrations are similar to normal due to Cbl binding to HC (Fenton and Rosenberg, 1989). The canine TC II has been studied

and showed similar molecular size , cobalamin binding activity, and immunologial reactivity as in human (Rappazzo and Hall, 1972).

TC II-mediated uptake of plasma Cbl has been shown in a number of cells (Hall 1975; Retief et al., 1966; Youngdahl-Turner et al., 1978), and Cbl is transported into cells via a plasma membrane TC II-Cbl receptor (TC II-R) expressed in many tissues (Bose et al., 1995a; Bose et al., 1995b). After endocytosis, the TC II-Cbl complex dissociates in endosomes or lysosomes, and free Cbl is released (Idriss et al., 1991). The free Cbl then enters the cytoplasm where it is reduced and converted to methyl-Cbl, or in mitochondria, where it is reduced and converted to adenosyl-Cbl. Methyl-Cbl and adenosyl-Cbl are used as cofactors for methionine synthase and methylmalonyl CoA mutase, respectively (Millman et al., 1977, Kolhouse and Allen, 1977). In addition to TC II-R, whose role in plasma transport of Cbl is well established, another protein known as megalin has been shown to bind to TC II-Cbl *in vitro* and mediate its endocytosis in BN/MSV cells (derived from yolk sac carcinoma) and perfused renal proximal tubules (Moestrup et al., 1996). The physiological significance of megalin-mediated TC II-Cbl uptake system in the plasma transport of Cbl is not certain. Due to the high expression in the apical membrane of kidney proximal tubules, renal megalin may function in the tubular reabsorption of TC II-Cbl, thus preventing Cbl loss in the urine.

Cobalamin malabsorption and Imerslund-Gräsbeck syndrome

Due to the widespread distribution of cobalamin in animal products (Sullivan LW, 1970; Smith A, 1962), Cbl deficiency usually occurs secondary to inherited or acquired defects of gastrointestinal Cbl absorption rather than dietary insufficiency. Inherited disorders of vitamin B_{12} include those in which the vitamin is not utilized by target cells, and those which are defective in absorption of the vitamin from the gut and transport to the appropriate tissues. The former includes a defect in the release of free vitamin B_{12} from lysosomes (CblF) (LaFramboise et al., 1992) and defects in the formation of one or both vitamin B_{12} cofactors (CblA-E). Defects of absorption and transport include intrinsic factor abnormalities (Spurling et al., 1964; Katz et al., 1974b; Yang et al., 1985), deficiencies of TC II (Hakami et al., 1971) Cbl F, and selective intestinal Cbl malabsorption.

Human Imerslund-Gräsbeck syndrome

Selective intestinal Cbl malabsorption is also called Imerslund-Gräsbeck syndrome (I-GS) which was independently described by Imerslund and Gräsbeck in 1960 (Gräsbeck et al., 1960, Imerslund O., 1960). Imerslund's ten patients were from six different families in south-east Norway, while Gräsbeck et al. reported two cases from Finland. The patients examined showed Cbl-responsive anemia and most of them also had mild proteinuria. Following thorough investigation, all known causes of Cbl deficiency were eliminated, including pathological intestinal flora, inactive intrinsic factor, and deficiency of

transcobalamin I or II. Both sexes were affected, and the inheritance was deemed recessive. The similarity of the two sets of cases suggested that they represented the same disease. Since then, totally 38 cases in Finland and 15 cases in Norway have been identified with selective Cbl malabsorption (Aminoff et al., 1995). More cases have also been reported in other countries. By 1969, 20 cases with I-GS had been diagnosed in Israel, and proteinuria showed predominantly albumin (Ben-Bassat et al., 1969). Other scattered cases were also described from the united state (Colle et al., 1961; Spurling et al., 1964), Denmark (Sievens CJ, 1964), Australia (Marsden et al., 1979), Turkey (Yetgin et al., 1983; Altay et al., 1995; 1999; Celep et al., 1996), and Saudi Arabia (Abdelaal and Ahmed, 1991). More recently a single case has also been reported in an Africa (Stones and Ferreira, 1999). By 1999, there were over 250 cases reported in the literature. The syndrome occurs worldwide, but its prevalence is higher in several Middle Eastern countries and Norway, and highest in Finland. The onset of signs of this disease is between 0 to 5 years of age, which mainly corresponds to the expected period of depletion of the child's vitamin B_{12} which was stored prenatally by transfer from the maternal blood. The clinical features of this syndrome include failure to thrive, infections, megaloblastic anemia, neuropathy and mild general malabsorption. Mild proteinuria is common. All the clinical features except Cbl malabsorption and proteinuria can be eliminated by parenteral, but not oral, Cbl administration (Broch et al., 1984). Linkage analysis of 38 patients diagnosed in Norway and Finland localized the unknown autosomal recessive megaloblastic anemia (*MGA*

1) gene to the short arm of chromosome 10 between markers D10S548 and D10S466 (Aminoff et al., 1995).

Due to the clinical heterogeneity of this syndrome among the affected families and complexity of the process of enterocyte transcytosis of Cbl, different putative molecular natures of Imerslund-Gräsbeck syndrome have been reported. One study showed lack of pathology by electron microscopy of ileal biopsies and normal IF-Cbl binding in mucosal homogenates and suggested the cause of this disease has a defect in intracellular trafficking of Cbl rather than a deficient synthesis of the receptor (Mackenzie et al., 1972). Another study in a different family showed defective uptake of Cbl by ileal mucosa *in vivo* (Burman et al., 1985). The difference between these two studies may be due to the different genetic defects, but is also possibly due to the same genetic defect but different study design.

Canine model with selective cobalamin malabsorption

A similar recessively inherited syndrome in giant schnauzer dogs has been reported, in which affected puppies exhibited inappetance and failure to thrive beginning between eight and twelve weeks of age. (Fyfe et al., 1989). Clinical laboratory tests demonstrated megaloblastic anemia, low serum Cbl concentrations, and mild proteinuria. Parenteral, but not oral, administration of cyanocobalamin corrected all clinical and laboratory abnormalities except proteinuria and Cbl malabsortption. Gastrointestinal cobalamin malabsorption was demonstrated by oral radiolabeled cobalamin administration, and

biochemical characterization of IF and TC II were normal (Fyfe et al., 1991a). Expression of IFCR was investigated in ileum and renal cortex of affected dogs by immunoelectron microscopy and cell fractionation, and the results demonstrated that there was no expression of IFCR in the apical brush-border membrane of either tissue (Fyfe et al., 1991b). Receptor-ligand binding assay showed IFCR activity in total homogenates was 3-4 fold higher than normal in ileal mucosal and one-tenth of normal in kidney of affected dogs. These data suggested that inefficiently expressed receptors accumulate in ileum but were rapidly degraded in kidney of affected dogs (Fyfe et al., 1991b).

Brush-border expression of IFCR is critical for intestinal IF-Cbl absorption. The defective functional expression of IFCR onto the apical membrane raised the consideration that IFCR may be a candidate gene for *MGA 1* in this family of dogs. The most common reason for disfuntional membrane expression of a membrane protein is mutation of the coding sequence, which results in abnormal protein folding (discussed later). Based on this phenomenon, we hypothesized that mutation of IFCR was the cause of selective intestinal Cbl malabsorption in this family of dogs.

The similarity of the clinical and laboratory features between human patients and the dog family with selective intestinal Cbl malabsorption suggested that this would be a good animal model for study of this syndrome. Due to the easy treatment of the disorder in humans, experimental samples are extremely hard to obtain from patients, greatly limiting investigation of the molecular mechanism of this disease. Biochemical study of IFCR in the affected dogs first

demonstrated the candidate gene for *MGA 1*. Further study with this model will enhance understanding of this syndrome and elucidate mechanisms of the cellular processing pathway of newly synthesized proteins.

Characterization of intrinsic factor-cobalamin receptor (IFCR)/cubilin

General characteristics of IFCR / cubilin

Clearly IFCR plays a key role in gastrointestinal Cbl absorption. Research has been done during the past over 25 years to further understand the function and molecular nature of this receptor. Solubilization of IFCR from distal small intestine has been carried out in guinea pig and pig (Katz et al., 1974a; Cotter and Rothenberg et al., 1976). Solubilized receptor had similar IF-Cbl binding characteristics to membrane-bound receptor, suggesting the functional domain of this receptor resided on the lumenal side of the enterocyte membrane.

Canine ileal IFCR purified by affinity chromatography revealed a 180 kDa protein on non-reducing SDS-PAGE, and 2-mercaptoethanol treatment produced two bands with apparent molecular weights of 59 and 42 kDa (Seetharam et al., 1981). Papain digestion prior to purification resolved IFCR into smaller subunits of the receptor which maintained IF-Cbl binding activity, both for dogs (Seetharam et al., 1982) and pigs (Kouvonen I, 1980; Kouvonen and Gräsbeck, 1980). Further cell fractionation of canine ileal enterocytes revealed an ~185 kDa IF-Cbl binding protein on brush-border membrane (Fyfe et al., 1991b). However, functional IFCR in the Caco-2 cell line, derived from a human colon carcinoma, was reported as a 230 kDa protein (Ramanujam KS, 1991a) which does not

dissociate into subunits upon disulfide bond reduction. This suggested that, instead of having a subunit structure, IFCR undergoes proteolytic cleavage once exposed to the ileal lumen with the resulting fragments being held together by disulfide bonds.

Besides the distal part of the small intestine, high levels of receptor for IF-Cbl were detected in human, canine, and rat kidney. The ratio of specific activity for kidney relative to intestine in these species was 116, 20 and 797, respectively (Seetharam et al., 1988). *In vivo* (Seetharam et al., 1992) and *in vitro* (Ramanujam et al., 1991b, 1992) functional studies of renal IF-Cbl receptor showed the same binding activity and immunoreactivity as the ileal receptor. Both activities were detected in the apical membrane of proximal tubule cells but not in distal tubule cells of the kidney. The size determined from SDS-PAGE for the renal receptor was reported as 230 kDa and was not reduced into subunits by reductive alkylation (Seetharam et al., 1988; Ramanujam et al., 1993a; Fyfe et al., 1991b). Quantitative amino acid analysis of the renal receptor gave a value of 457,310 g of amino acid / mol of IF-Cbl binding activity but was thought to represent a dimer (Seetharam et al., 1988).

All the ileal and renal IFCR data together suggested that IFCR undergoes proteolytic cleavage once exposed to the ileal lumen rather than having a subuit structure. This hypothesis is based on the assumption that ileal and renal IFCR are the same gene products. IF is not thought to enter the circulation system during ileal endocytosis. The presence of IFCR in the kidney suggested that renal IFCR had functions other than renal IF-Cbl reabsorption.

In the meantime, the study of an independent group described a 280-kD protein (gp280), defined by monoclonal antibodies raised against rat renal brush-border, which was found in the intermicrovillar domain of the proximal tubule cells of kidney and rat yolk sac epithelium (Sahali et al., 1988). Antibodies to gp280 induced fetal malformations via trapping of the target antigen in the early endocytic compartment thus preventing its normal function in lysosomal transfer (Le Panse et al., 1994; 1995). The function of gp280 was thought to be a receptor, but its ligand(s) was not known until 1997. Rat renal gp280, like the IFCR, binds to the IF-Cbl complex with an association constant of $0.3 \times 10^9\ M^{-1}$ and mediates its internalization (Seetharam et al., 1997). In addition, antibodies against purified gp280 and IFCR can inhibit the binding of IF-[^{57}Co]Cbl complex to intestinal, renal, and yolk sac apical membranes and revealed a single identically sized protein on immunoblotting of renal membranes, suggesting functional and immunological identity of the two proteins. The similar mobility on SDS-PAGE and restricted tissue distribution of gp280 and IFCR suggested the possibility that IFCR and gp280 were similar or identical proteins.

IFCR/gp280 was then considered an over 200 kDa protein facilitating the intestinal endocytosis of IF-Cbl and renal reabsorption of some unidentified proteins. In late 1997, using receptor-associated protein (RAP) affinity chromatography, an IF-Cbl binding renal epithelial protein of ~460 kDa was co-purified with the TC II-Cbl binding 600 kDa receptor, megalin (Birn et al., 1997). The IF-Cbl binding activity was further confirmed by IF-Cbl affinity chromatography, which eluted the same ~460 kDa protein from rabbit and human

kidney homogenate. *In vitro* binding analysis demonstrated a calcium-dependent high affinity binding of IF-Cbl to a site distinct from the RAP binding site. Electrophoretic mobility and immunoreactivity was compared between rat gp280 and rabbit ~460 kDa IF-Cbl-binding protein, and the two proteins showed identical SDS-PAGE migration and immunoreactivity. Microscopic autoradiography revealed the ~460 kDa protein in both kidney and terminal ileum and *in vivo* microinjection demonstrated the facilitation of the endocytosis of IF-Cbl by this membrane protein.

Molecular cloning of human and rat cubilin

The molecular characterization of this ~460 kDa protein (IFCR/gp280) was not solved until its recent cDNA cloning from rat and human (Moestrup et al., 1998; Kozyraki et al., 1998). The cDNA clone corresponds to a single transcript of 11.6 kb in intestine, kidney and yolk sac tissues in rat, where IFCR/gp280 is expressed. Using fluorescence *in situ* hybridization, radiation hybrid mapping and screening of YAC clones, the human cDNA clone was mapped on the short arm of chromosome 10 (10p12-p14) (Kozyraki et al., 1998), which is in the same region as human MGA 1. These results suggested that this ~460 kDa protein was the candidate disease-causing gene product for selective intestinal Cbl malabsorption in some patients. All these data showed that the newly purified protein had the same ligand binding activity, tissue distribution, and immunoreactivity, but had different molecular weight compare to IFCR/gp280. Since two very well characterized markers bigger than 460 kDa were used in the

most recent study (Birn et al., 1997), the previous over 200 kDa molecular weight for IFCR/gp280 was possibly misjudged by relying on migration on SDS-PAGE with 200 kDa as the highest molecular weight marker.

The assembled cDNA revealed an uninterrupted open reading frame of 10.8 kb and a deduced 3621-3623 amino acid protein for human and rat, respectively. The overall homology between the encoded proteins of these two species is 69%. The predicted domain organization of this protein contains an N-terminal stretch of approximately 110 amino acids with no apparent homology to known proteins, a cluster of eight EGF (epithelial growth factor) type B repeats and 27 contiguous CUB domains (Moestrup et al., 1998; Kozyraki et al., 1998)(Fig 1.2). Due to the unique tandem arrangement of CUB domains in the protein sequence, the ~460 kDa protein (IFCR/gp280) has been designated as **cubilin,** which will be used for the remainder of this thesis.

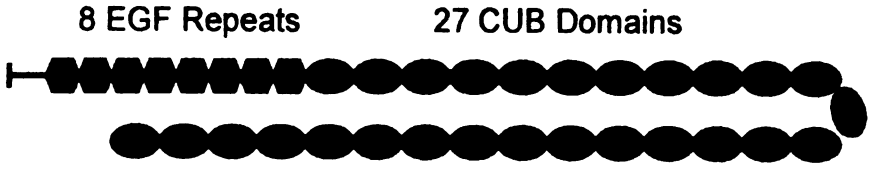

Figure 1.2 Predicted protein structure of cubilin

More evidence suggests that the protein expressed in intestine and kidney are the same gene product. An early study (Robin et al., 1974) reported that a patient with IG-S had an inherent renal proximal tubular defect in amino acid reabsorption which was not Cbl deficiency related. The patients with selective Cbl malabsorption with proteinuria have reduced urinary IFCR activity compared

to the normal control (Dugue et al., 1998). Further genetic studies with 17 Finnish families with selective intestinal Cbl malabsorption identified two independent disease-specific mutations in the cubilin gene (*CUBN*) (Aminoff et al., 1999). One was a missense mutation (FM1) apparently in the IF-Cbl binding site (Kristinasen et al., 1999). The other mutation was a C to G mutation activating a cryptic intronic splice site and introducting multiple stop codons in the mutant transcript (FM2). These mutations indicated that defects of cubilin in the intestine caused Cbl malabsorption. The Finnish patient with the FM2 mutation also secreted dramatic amounts of urinary alipoprotein A-I (apoA-I) which was not detected in the normal control or FM1 patient (Kozyraki et al.,1999). ApoA-I was also detected in urine of dogs (five of six) with selective intestinal Cbl malabsorption, caused by disfunctional expression of IFCR on both intestinal and renal epithelial cell membranes, but was not detected in normal control dogs. These results elucidated that a null mutation in *CUBN* (FM2) affects cubilin functions in both intestine and kidney and elegantly supports the hypothesis that these two proteins are the same gene product.

The patient with apoA-1 proteinuria indicated that besides IF-Cbl complex, apoA-1 is another ligand for cubilin which can be reabsorbed in the kidney. *In vitro* studies, such as cubilin affinity chromatography and surface plasmon resonance analysis, further demonstrated the high-affinity binding of apoA-I and high-density lipoprotein (HDL) to kidney cubilin (Kozyrak et al., 1999; Hammad et al., 1999; 2000). Cubilin-expressing yolk sac cells showed efficient ^{125}I-HDL endocytosis that could be inhibited by antibodies against apoA-I and cubilin.

Other ligands have also been defined for renal cubilin recently, such as myeloma light chains (Batuman et al., 1998) and albumin (Birn et al., in press).

Both human and rat cubilin open reading frame begin with a 24-20 amino acid signal peptide which facilitates binding of the translating ribosome to bind the endoplasmic reticulum (ER) and starts the membrane protein biosynthetic pathway. An Arg-X-Arg/Lys-Arg consensus sequence can be found following the signal peptide in both sequences, which is a site cleaved by the trans-Golgi proteinase furin (Kozyraki et al., 1998; Moestrup et al., 1998). Cubilin N-terminal sequence starts just after furin site. This indicates that the receptor is synthesized as a precursor undergoing further proteolytic processing in the trans-Golgi. Two of the EGF repeats contain the discontinuous consensus sequence for Ca^{2+}-binding, which may be required for a particular conformation for ligand binding. The CUB domains, named as a structural feature common to complement subcomponents **C**1r/C1s, the Xenopus patterning protein, **U**vs.2, and **B**one morphogenic protein-1, have ~110 residues with two disulfide bridges, and bind a diverse array of protein, carbohydrate, and phospholipid ligands (Bork and Beckmann, 1993). CUB domains are common to an increasing number of developmentally expressed proteins, but none of the known proteins contains more than 5 CUB domains. The extensive repetition of CUB domains provides another indication that this ~460 KDa is a multiligand receptor with multiple potential binding sites.

Except for the leader peptide, no sequence compatible with a transmembrane domain or glycosylphosphatidylinositol (GPI) anchor-addition

signal could be identified in cubilin. This excludes the possibility that the protein is a transmembrane protein or GPI anchored protein that is synthesized with a cleavable hydrophobic C terminus. Early studies with guinea pig ileum homogenate without detergent treatment showed the receptor is soluble and maintained IF-Cbl binding activity (Cotter et al., 1976). Later study confirmed that association between the cubilin and membranes is weak since approximately 50% of cubilin could be released into the fluid phase during simple mechanical grinding without detergent or during incubation of membranes with heparin or EDTA (Moestrup et al., 1998). All these data suggested that cubilin is a peripheral membrane protein.

As a peripheral membrane protein without internalization signals, how cubilin can facilitate the endocytosis of IF-Cbl complex is an open question. Co-internalization of a receptor that lacks internalization signal(s) by means of another receptor has been shown previously. Thus, the glycosylphosphatidylinositol-anchored urokinase receptor is endocytosed by coupling of urokinase receptor-bound urokinase-inhibitor complex to low density lipoprotein receptor-related protein (LRP) (Nykjaer et al., 1997, Herz et al., 1992). Megalin, which is a closely related to LRP (both are low-density lipoprotein receptor family members), also binds the urokinase-inhibitor complex (Moestrup et al., 1993), and may perform a similar function in regard to both the urokinase receptor and cubilin-ligand complexes. *In vitro* study showed high affinity interaction (K_d~7nM) between cubilin and megalin which does not interfere with subsequent IF-Cbl binding to cubilin (Moestrup et al., 1998; Hammad et al.,

2000). *In vivo* immunohistochemistry of kidney, intestine and yolk sac in rat showed colocalization of these two proteins in these tissues (Sahali et al., 1988, 1993, Birn et al., 1997, Moestrup et al., 1998). Megalin has a single transmembrane domain and cytoplasmic domain motifs that signal for coated pit-mediated internalization. These data suggest that megalin might mediate cubilin endocytosis and recycling to the plasma membrane. Since the *in vitro* cubilin-megalin complex is stable at pH 5, the two receptors might remain in complex during the entire recycling pathway (Fig 1.3).

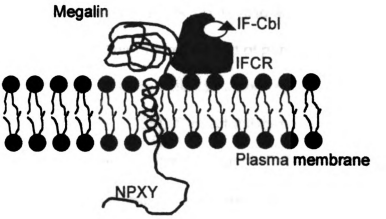

Figure 1.3 Schematic representation of the interaction between peripheral membrane protein cubilin and transmembrane protein megalin.

Functional domains of cubilin

To better understand the relationship between cubilin function and its structure, a mammalian expression approach has been used to identify the cubilin regions important for membrane association and ligand binding

(Kristiansen et al., 1999). The results showed the N-terminal cubilin region is crucial for membrane association. This region has no classical transmembrane segment, but helical plotting demonstrated a conserved amphipathic helix motif that may be involved in the assembly of cubilin (rat amino acid 74-109, human amino acid 73-108). A recent electron microscopic study of purified bovine cubilin has shown the formation of trimers in solution without detergent or lipid (Lindblom et al., 1999). A region with four-heptad repeats, actually a part of the putative amphipathic helix structure, has been proposed to account for the assembly. The only cysteine located in the 110-amino acid N-terminal sequence, outside of the CUB domains and EGF repeats, might also account for the partial disulfide bond-dependent dimerization of a minor part of purified receptors.

The binding site for IF-Cbl has been located in the region encompassing CUB domains 5-8 (Kristiansen et al., 1999). This establishes the CUB domain structure as a ligand-binding domain. The two mutations identified in the human cubilin gene both relate to the critical region now known to harbor the IF-Cbl binding region. The FM2 mutation, suggested to activate a cryptic intronic splice site leading to truncation of the receptor in CUB domain 6, may lead to an inactive or unstable translation product, whereas the P/L substitution (FM1) in CUB domain 8 might cause a structural change specifically impairing IF-Cbl binding. The CUB domain is a barrel-like structure containing two layers of five-stranded β-sheets with the β-turns in a surface-exposed position as in the antigen binding regions of immunoglobulins (Menendez et al., 1995; Dias et al., 1997). In view of the existence of 27 CUB domains in cubilin, a high number of

putative sites for various protein interactions may then be predicted.

A site for binding of RAP was localized within CUB domains 13-14. The physiological importance of this interaction remains to be determined, but it is tempting to speculate that RAP may assist the processing/folding of cubilin in line with its suggested chaperone function which is important for normal processing of the LDL receptor-related protein and megalin (Willnow et al., 1995). RAP, a 40-kDa endoplasmic reticulum protein, serves as a novel kind of chaperone or escort protein and protects multiple ligand binding sites of processed low density lipoprotein receptor family proteins, in particular the two giant receptors megalin and LRP. The association with RAP indirectly indicates that cubilin is a multiple ligand binding protein.

Regulation of cubilin expression in small intestine:

Most gastrointestinal functions are not evenly distributed along the intestinal tract. The remarkable degree of coordination between the development of various aspects of gastrointestinal function suggests that the process may be triggered by a single or a few central mechanisms, such as weaning and (or) hormones (Hermiston and Gordon, 1995). Early studies showed the gradient of the enzymatic activity, or even the absorptive capacities for sugar and amino acids, correlated with the gradient of mucosa mass of the small intestine (Laganière et al., 1984). However, later studies on the regulation of sucrase isomaltase (SI) and aminopeptidase expression along small intestine showed the translational or post-translational regulation of these enzymes, but no change of the mRNA level (Hoffman and Chang, 1991; Danielsen et al., 1986). Different

genes show different regional specificity and are under different regulatory mechanisms.

During fetal development of the human intestine, cubilin activity can be detected along the entire length of intestine in early fetal development, but is located to the distal ileum by the end of fetal development (Schohn et al., 1992). Studies in rats also suggested that cubilin expression was developmentally regulated (Ramasamy et al., 1989; Ramanujam et al., 1993b). The functional expression of cubilin in distal small intestine was confirmed by oral administration of radio-labeled Cbl in dogs and humans (Baker et al., 1958; Fleming et al., 1962; Hagedorn and Alpers, 1977). The results of surgical transposition of ileal segments from distal to proximal part of the canine intestine further confirm the ileal location for IF-Cbl absorption in dogs (Drapanas et al., 1963). The distribution of cubilin along the gastrointestinal tract is against the gradient of the mucosa mass for the dog (Laganière et al., 1984). The regional specific expression of cubilin along the small intestine must be regulated at some level. There is some *in vivo* evidence to suggest that the ileal capacity for Cbl absorption is regulated by modulation of cubilin expression during pregnancy (Brown et al., 1977; Robertson and Gallagher, 1983), but whether it is regulated by certain hormones needs to be investigated.

From another point of view, the lining epithelium of the small intestinal mucosa is in a constant and rapid state of renewal (Weinstein WM, 1974; Leblond CP, 1980; Stappenbeck et al., 1998). Newly formed enterocytes arise in the crypt region and migrate up the villus to be shed into the intestinal lumen at

or near the villus tip. The overall process of cell renewal depends on the number of cells of the proliferative compartment; the rate of cell proliferation; and the migration time. During this migration, enterocytes mature and differentiate for a host of specific functions. Immunoelectrophoretic studies on human small-intestinal brush-border proteins showed the amounts of lactase, sucrase, maltase, microvillus aminopeptidase and dipeptidyl peptidase IV from jejunum biopsies had their maximum activities near the mid-region of the villi and their lowest activities at the base of the crypts (Skovbjerg H, 1981). How different gene expression along crypt to villus axis is regulated is still an unsolved question. One of the most well characterized gene regulatory mechanisms along the crypt-villus axis is the SI gene for which transcriptional regulation at the promoter depends on at least two groups of transcriptional proteins: hepatocyte nuclear factor 1(HNF-1) and caudal-related homeodomain proteins (Cdx)(Wu et al., 1994; Suh et al., 1996).

An immunoelectron microscopic study (Levine et al., 1984) of canine cubilin distribution during enterocyte maturation from crypt to villus tip of ileal mucosa showed that synthesis of cubilin can be easily detected in the mid-villus cells, with the majority distributed intracellularly. Expression of cubilin dramatically increases in the villus tips, where also the majority of cubilin can be detected in the microvillus pits. The expression of cubilin is extremely low in the crypt cells. Later study (Kapadia and Essandoh, 1988) with enriched villus and crypt cell populations also showed IF-Cbl binding was about 30-fold greater in ileal villous cells than in crypt cells. From the above, it is evident that during cell

differentiation from crypt to villus, cubilin expression is up regulated during differenciation (maybe just slow). Although the particular signals affecting cubilin expression remain unknown, we can evaluate normal cubilin expression in different tissues, different regions of the same tissue, and the same region at different stages of cell differentiation to determine how cubilin expression is regulated. This will provide the basis for further studies of cubilin regulation and functions.

Protein folding in the secretory pathway

Being a membrane-associated protein, cubilin, like other secretory and membrane proteins, is likely carried to the plasma membrane via a specific membrane protein biosynthetic transport pathway. The first and most important compartment of this pathway is the endoplasmic reticulum (ER) which is responsible for protein synthesis, early posttranslational modifications, maturation and export of the fully folded protein to the destined location. Among all of these functions of ER, one of the most notable are mechanisms to monitor the fidelity of early biosynthetic events in the protein export pathway, also known as "ER quality control" (Kopito RR, 1997). This quality control is critical for normal cell function since it prevents premature export of incompletely or improperly folded proteins from ER.

In recent years, numerous inborn errors of metabolism have been identified which affect secretory or plasma membrane proteins (Medeiros-Neto et al., 1996; Repaske et al., 1996; Kuivaniemi et al., 1991; Lehrman et al., 1987;

Yang et al., 1993). In many instances, mutations causing minor changes in proteins that result in a folding defect lead to abnormal protein trafficking, suggesting that proper conformation is critical for protein transport as well as biological activity. During protein biogenesis, a peptide chain can self-assemble into a stable, low free-energy conformation based on information contained within the primary structure. For many proteins, additional factors are required for proper folding. All these factors reside within the ER and are known as ER chaperones. According to their specific functional mechanism, some of them have been defined as primary controls and apply to all newly synthesized proteins. The others, defined as secondary controls, are specific for a particular protein or protein family (Ellgaard et al., 1999).

The synthesis of primary molecular chaperones, including Bip, GRP94, and calnexin, etc., can be induced in response to the accumulation of unfolded secretory proteins (Chapman et al., 1998). This induction can be at the transcriptional level or posttranslational level. Because ER chaperone associations are based on recognition of features enriched in incompletely folded versions of exportable proteins, the Ca^{2+} and ATP dependent associations of ER chaperones are usually at their highest levels immediately upon nascent chain translocation into the ER, and terminated before export of the folded proteins from their compartment (Sambrook JF, 1990; Clairmont et al., 1991). The fact that molecular chaperones act on "substrate" proteins does not affect self-assembly. By interacting with nascent chains, chaperones prevent undesirable

protein-protein interactions and increase the chances that newly made proteins will have the opportunity to achieve their native structure.

Primary control also includes disulfide isomerase and prolyl isomerase. They are the true foldases, in that they catalyze thiol-disulfide interchange with broad substrate specificity (Freedman, 1995). As the nascent chain enters the ER lumen, the rapid interaction within the hydrophobic domains is accompanied by the ordered formation of intramolecular disulfide bounds, which stabilize secondary and tertiary structures and can be critical for maintaining a biologically active conformation. Within the oxidizing ER environment, reactive thiols also can form mispaired disulfide bonds, and subsequent correction of aberrant disulfide bonds may represent one of the rate-limiting steps in protein folding. Specific cysteine residue mutations can also cause severe disulfide bond misformation which eventually blocks protein transportation (Reddy and Corley, 1998).

Another important primary control mechanism involves oligosaccharide trimming (Parodi AJ, 1999). A 14-saccharide unit, which contains two N-acetyl glucosamine (GlcNac) residues, nine mannoses and three glucose resides, is initially added to asparagine-linked consensus acceptor sites (NXS/T) in glycoproteins. During ER enzyme-mediated trimming, the interaction between nascent proteins and ER chaperones (calnexin) are regulated by trimming enzymes, such as glucosyltransferase and glucosidase (Trombetta and Parodi, 1992; Sousa et al., 1992), which serve as folding sensors to direct the unfolded glycoprotein to ER chaperones. Thus, proper N-linked glycosylation and oligosaccharide trimming are also important for nascent protein folding.

Quite a number of protein specific factors that are responsible for effecting folding and assembly of nascent proteins have been identified in different species (Ellgaard et al., 1999). A well-studied factor, classified as an escort protein is receptor associate protein (RAP), the function of which was first recognized by its interaction with LRP molecules through the whole biosynthetic pathway, even remaining associated on the cell surface (Bu et al., 1995; Farquhar et al., 1995). Later study showed that RAP can also interact with other membrane receptors, like megalin (Moestrup et al., 1996) and cubilin (Moestrup et al., 1998). Absence of RAP can cause the defective transport of LRP through the ER (Willnow et al., 1995). With RAP association, both RAP and LRP can exit the ER, which suggests that the interaction maintains a favorable conformation and prevents premature association of LRP ligands in the ER, which could lead to receptor retention and /or degradation. RAP dissociation occurs when LRP reaches the cell surface and LRP ligands can displace the escort. Surface expression of RAP has been questioned in a study which demonstrated that RAP relocates to RAP binding site *in vitro* when tissues are snap frozen (Abbate et al., 1993).

Cubilin is a large protein which contains 76 disulfide bonds and 47 potential N-glycosylation sites in human (Kozyraki et al., 1998). The assembly process within the ER must be time consuming and require a lot of ER chaperones. The *in vitro* interaction with RAP indicates that RAP or other proteins may act as chaperones to facilitate the intracellular maturation. It is not known whether cubilin-specific chaperones are involved in its processing.

Biochemical studies of affected dogs with selective intestinal Cbl malabsorption demonstrated the accumulation of cubilin in the ileal enterocytes, but rapid degradation in the kidney epithelial cells (Fyfe et al., 1991b, Fyfe, unpublished data). The different fates of the same protein in different tissues suggests that there is tissue-specific machinery for the protein assembly and export.

The mechanism of defective expression of cubilin on the apical brush-border membrane of both ileal and kidney epithelial cells of affected dogs with selective intestinal Cbl malabsorption is the major focus of this project. The most common reason for an ER export disorder is mutation in the coding sequence of the exportable protein, eventhough defects of other accessory proteins may also be involved. The latter case has been demonstrated only in abetalipoproteinemia (Wetterau et al., 1992), a defect of the apo B-100 specific processing enzyme, microsomal triglyceride transfer protein.

General outline for this thesis

A dog family with selective intestinal cobalamin malabsorption, which closely resembles the human Imerslund-Gräsbeck syndrome, provides a valuable genetic model to further investigate the mechanism of this inherited disease. The general goals of this thesis were to clone the candidate disease-causing gene, cubilin / intrinsic factor-cobalamin receptor (IFCR), and characterize its gene product. To achieve these goals, three closely related projects were conducted and are described in this thesis: (1) Cloning of the full-

length cDNA of canine cubilin, providing the molecular basis for further study of this candidate gene; (2) Studies on cubilin expression and post-translational modification in the canine gastrointestinal tract, which helped to better understand this newly cloned gene and the relationship between this gene and the disease in the dog family; and (3) Linkage analysis between the disease locus and candidate gene loci, which efficiently allowed determination of whether two candidate loci habored a disease-causing mutation.

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CHAPTER 2

CLONING OF CANINE CUBILIN

Introduction

Gastrointestinal absorption of vitamin B_{12} (also called cobalamin,Cbl) is essential for animals, including humans, to maintain normal hematopoiesis and integrity of the central nervous system. Cbl absorption is a complex process and requires many Cbl binding proteins. A defect of any one of these proteins can cause Cbl deficiency. Cubilin, previously known as intrinsic factor-cobalamin receptor (IFCR), plays a critical role in endocytosis of the IF-Cbl complex in distal small intestine and reabsorption of various proteins in the kidney.

A family of dogs with selective intestinal Cbl malabsorption (Fyfe et al., 1989) has been identified that has similar clinical and laboratory features as seen in humans with a disorder known as Imerslund-Gräsbeck syndrome (I-GS) (Gräsbeck et al., 1960; Imerslund O., 1960). The affected puppies exhibited failure of gastrointestinal absorption of oral radiolabeled Cbl. Immunoelectron microscopy and cell fractionation studies demonstrated a defect in cubilin expression on apical brush border membranes of ileum and renal proximal tubules, whereas other brush-border proteins were expressed normally (Fyfe et al., 1991a, b). Purified normal and affected dog renal cubilin showed similar migration on SDS-PAGE, but affected dog cubilin had abnormal proteolytic peptide profiles and the asparagine-linked oligosaccharides were endoglycosidase H-sensitive. The cubilin of affected dogs accumulated in ileal enterocytes but was rapidly degraded in the renal epithelial cells. All these findings suggested that affected dog cubilin did not fold properly and did not reach the mid-Golgi compartment of the biosynthetic pathway, most likely being

retained in the endoplasmic reticulum (ER). Many such so-called ER storage diseases which selectively inhibit cell surface expression of a plasma membrane or secretory protein have been described (Moolenaar et al., 1997; Medeiros-Neto et al., 1996; Repaske et al., 1996; Kuivaniemi et al., 1991; Lehrman et al., 1987; Yang et al., 1993). With the exception of abetalipoproteinemia (Wetterau et al., 1992), these disorders have been attributed to mutations in the coding sequence of the exportable protein.

Rat (Moestrup et al., 1998) and human (Kozyraki et al., 1998) cubilin have recently been cloned and showed a unique tandem arrangement of 8 EGF domains and 27 CUB domains. Two independent mutations have been identified in *CUBN* in 17 Finnish families with IG-S (Aminoff et al., 1999). To further characterize the canine model with selective intestinal Cbl malabsorption and to understand the multiple functions of the gene, we cloned the canine cubilin cDNA from dog kidney proximal tubule epithelial cells and compared the canine cubilin sequence to human and rat sequences.

Materials and Methods

Peptide sequencing of purified canine renal cubilin

Canine renal cubilin was purified by IF-Cbl affinity chromatography as previously described (Fyfe et al., 1991b) and transferred from SDS-PAGE gel to PVDF membrane. *In situ* endoLys-C or trypsin digestions, separation of peptides by microbore HPLC, and peptide sequencing were performed by the Protein Chemistry Facility of the Worcester Foundation for Experimental Biology (Shrewsbury, MA).

Canine kidney cDNA library screening

A canine cDNA library was constructed from RNA isolated from normal canine pooled kidney proximal tubular epithelial cells, using Trizol (Life Technologies, Bethesda, MD) according to the manufacturer's instructions. Total RNA was sent to Stratagene (La Jolla, CA) for construction of the library in the bacteriophage lambda vector, Lambda Zap II, using both random octamer and oligo dT priming of the cDNA synthesis reaction. This library was screened with a digoxigenin-labeled probe derived from the rat cubilin cDNA (a kind gift of Pierre Verroust, INSERM, Pairs, FR). Hybridizing plaques were purified, and the 1.6 kb cDNA insert of one (DX2) was sequenced, revealing 85% identity with the human cubilin sequence. DX2 was labeled with [^{32}P]α-dATP to high specific activity using a random primer labeling kit (Boehringer Mannheim) and used as a probe to hybridize to 6 Hybond™-N nylon membrane (Amersham Pharmacia Biotech) lifts totaling 3X10^5 pfu from the canine kidney proximal tubule epithelial cDNA

library. Duplicate membrane sets were prehybridized in 6X SSC, 0.5% SDS, 5X Denhardts solution, 20mM NaH_2PO_4, and 100 μg/ml sonicated sperm DNA for 4 hrs at 65°C. Hybridization was performed in the same solution after adding the denatured probe for 16 hours at 65°C. Membranes were washed twice with 6X SSC at room temperature and twice with 2XSSC and 0.5% SDS for 30 min at 68°C. Positively hybridizing plaques were picked and rescreened using the same probe for three more rounds. The true positive plaques were purified, and *in vivo* excision of the phagemid pBluescript (SK-) was performed according to Stratagene protocol. XL1-Blue bacteria were infected with the positive phagemids and grown on LB agar plates containing 50 μg/ml ampicillin. Plasmid DNA was purified from overnight MR 2000 cultures (MacConnell Research, San Diego CA) using QIAGEN's plasmid purification kit (QIAGEN Inc. Santa Clarita CA).

PCR amplification of cubilin cDNA probes for sequential screening:

Sequence information obtained from the cDNA clones was used to design primers, F3922, 5'-CAGGCAACAACTGGCAACACGGT-3', R4907, 5'-GCAGGGAACAATGGAGAGGATA-3', for use in PCR reactions to amplify a cDNA probe for sequential rounds of screening. The fifty μl final reaction volume contained: 1X PCR buffer (Gibco BRL), 0.25 mM each dNTP, 2 mM $MgCl_2$, 0.25 μM each primer, 0.4 ng plasmid DNA template and 2.5 units Taq DNA polymerase (Gibco/BRL). After a hotstart denaturation at 95°C for 5 min, the reactions were carried out for 10 cycles of denaturation at 94°C for 1 min,

annealing at 62°C for 1 min, and extension at 72°C for 5 min. Then the annealing temperature was decreased to 58°C for 26 more cycles. The amplified fragments were size fractionated by agarose gel electrophoresis in 1X TAE and were gel purified to use as probes for the next round of screening. The screening was carried out using the same protocol and same amount of cDNA library as above.

For a third round of screening, 5 micrograms of canine kidney cortex total RNA were reversed transcribed with oligo dT primer (Gibco) at 42°C to synthesize first strand cDNA. 2 μl of the RT product was used as template to amplify a cDNA probe using primers F5404, 5'-GGTCACTTGGTGGGGC-GATACT-3' and R6066, 5'-GTCCAGGGAAAGGATGTTGAGTT-3' designed from the 3' end sequence of a newly cloned fragment. The PCR reactions were performed under the same conditions as above for 31 cycles except using 67°C annealing for 20s and 72°C extension for 2 min. The PCR product was gel purified and used as probe to screen the canine cDNA library again.

In multiple rounds of screening with newly isolated insert cDNAs as probes in each round, 10 clones (DX2-DX11) containing overlapping inserts were selected and partially or fully sequenced on both stands. Most were sequenced by primer walking, but DX11, containing an ~5 kb insert was sequenced using an *in vivo* transposon-based kit for random insertions of primer sites (GPS-Genome Priming System, New England Biolabs, Beverly, MA). All sequences were generated by dideoxy termination cycle sequencing methods on an ABI 373A Automated Sequencer (Applied Biosystems, Inc, Foster, CA). cDNA sequences were assembled and analyzed using DNASTAR (DNASTAR, Madison, WI) or

SEQUENCHER software (Gene Codes Corp, Ann Arbor, MI) and have been submitted to GenBank (accession #AF137068). Position 1 of the canine cubilin cDNA refers to the first nucleotide of the full-length cloned cDNA. By this system, the A residue of the first ATG is at position 74.

Results

Cloning of Canine Cubilin cDNA

Using a single rat cubilin cDNA fragment to screen a λZap cDNA library derived from dog kidney proximal tubule epithelial cells, an initial 1.6 kb clone encoding a portion of canine cubilin was identified. Two independent clones which covered upstream and downstream sequences were identified using this 1.6 kb fragment as probe to screen the same canine cDNA library. A number of other clones were identified using polymerase chain reaction-generated probes for further screening. Fig. 2.1 schematizes five overlapping clones used to construct the final cDNA. Canine cubilin cDNA contains11,282 bp including 73 bp of 5' and 349 bp of 3' untranslated sequence. There was a poly A addition signal sequence 320 bp 3' of the stop codon and 21 bp 5' of the beginning of the poly A tail. The nucleotide sequence was 85 % identical to the human and 74 % identical to the rat cubilin sequences in GenBank.

Primary structure of canine cubilin:

The open reading frame (ORF) of 10,860 bp had a deduced amino acid sequence of 3,620 residues representing a 397,435 Da protein (Fig. 2.2). Alignment of the deduced amino acid sequence demonstrated overall amino acid identity of 83 % with human and 70 % with rat cubilin. The primary structure of canine cubilin, which contains a 110 amino acid N-terminal sequence; 8 EGF

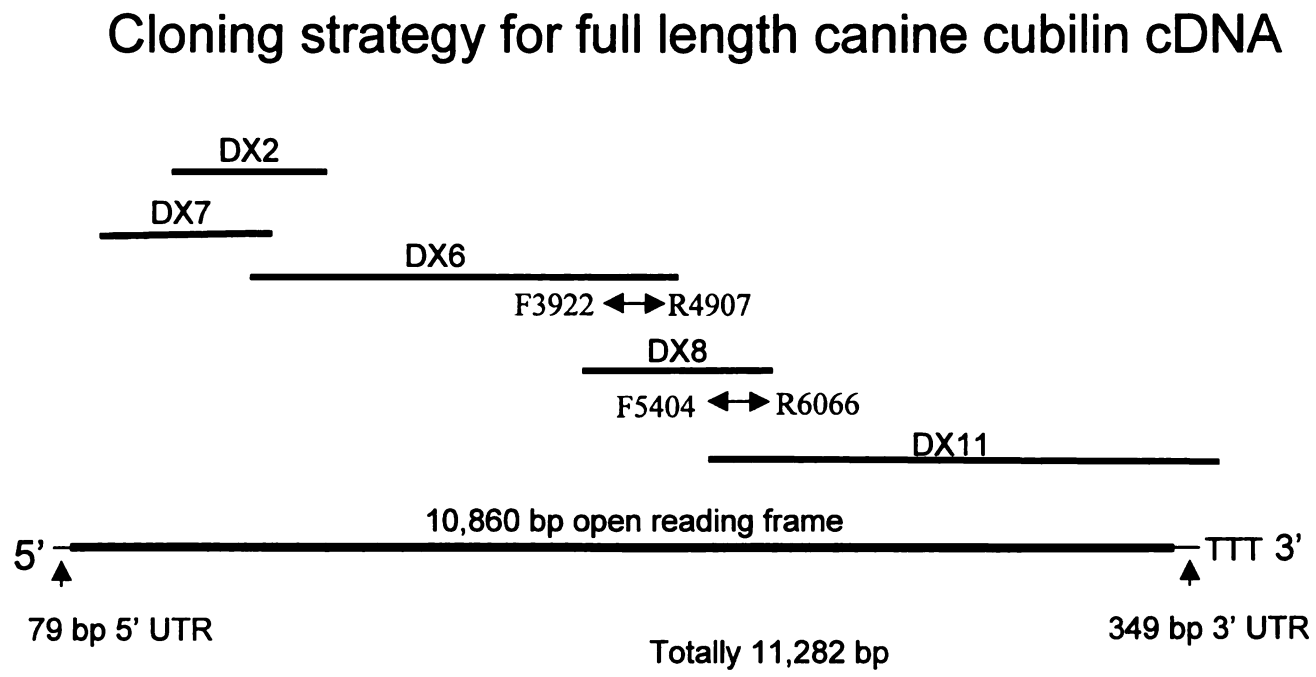

Figure 2.1: cDNA cloning of canine kidney cubilin. The 11,282 bp cDNA sequence encoded by five clones. DX2:464-2059; DX6: 1281-5056; DX7:1-1395; DX8: 3835-6340 and DX11: 5844-11282. The cDNA clone contains 73 bp of 5'and 349 bp of 3' untranslated sequence. There was a poly A addition signal sequence 320 bp 3' of the stop codon and 21 bp 5' of the beginning of the poly A tail. The nucleotide sequence was 85% identical to the human and 74% identical to the rat cubilin sequences in GenBank.

Figure 2.2: Primary sequence of canine cubilin as deduced from cDNA clones. Deduced amino acid sequence of 3,620 residues representing a 397,435 Da protein. Alignment of the deduced amino acid sequence demonstrated overall amino acid identity of 83% with human and 70% with rat cubilin. The arrow shows the furin cleavage site. The underlined sequence represents the consensus sequence Arg-X-Arg/Lys-Arg for cleavage by the trans-Golgi proteinase furin. The sequences verified by protein sequencing of purified canine kidney cubilin are shaded.

MSSPFLWSLIILLTFAESNGEAGGFELQRQKRSIDFQQPRMATERGNLVFLVGSAQNIEFRTGSLGKIKLN
EEDLGECLHQIQKNKEDITDLKRSAVNVPQNISSQIHQLNSKLVDLERKFQSLQQTVDKKVCSSNPCQNGG
TCLNLHDSFFCICPSQWKGPLCSVDVNECQIYSGTPLGCQNGATCENTAGSYSCLCSPETHGPQCASKYDD
CEGGSKALCVHGICEDLVRVKADEPKYNCICDAGWTSPLNSSACVLDIDECNLQHAPCSPLVQCFNTQGSF
YCGACPTGWQGNGYSCQDIDECKINNGGCSVVPPVMCVNTLGSYHCQACPPGYQGDGRVCTVIDICSVNNG
GCHPEASCSSVLGSLPLCTCLPGYTGNGYGPNGCAQLSDTCLSHPCLNGQCIETVSGYLCKCESGWAGINC
TENINECLSNPCFNGGTCVDGVNAFSCECTRFWTGFLCQIPQQVCGGSLSGMDGSFSYMSPDVGYVHDVNC
FWVIRTEDRKVLRITFTFFQLESVNNCPHEFLQIHDGDSSAALQLGRFCGSVLPHELLSSNNALYFHLYSE
HFRSGRGFTIRWETQQPECGGILMGTYGSIKSPGYPGNYPPXRDCVWQVVTSPDLLITFTFGTLSLEHHDD
CSKDYLEIRDGPLYQDPSLGKFCTTLSVPPLQTTGPFARVHFHSDNQINDQGFHITYLTSPSDLHCGGNYT
DPEGLLSSDLSGPFTHNRQCIYIIKQPLGEQIQVNFTHVELEGQSSCSQSHIEVRDDKILLGKVCDNETLP
HIKSIRNHIWIRLKIDASLVRASFRAVYQVACGGELTGEGVIRSPFYPNVYPGERICRWTIHQPQSQVVIL
NFTAFGIESSAHCDTDYIEIGSSSILGSPENKKYCGTDIPLFITSVYNFLYVIFVKSSSTENHGFMAKFSA
ADLACGEILTESTGIIQSPGHPNIYPHGINCTWHILVQPGHLIHLIFRKFHLEFHYNCTNDYLEVYDTGSN
TYLGRYCGKSIPPSLTSSTNSLKLIFVADSDLAYEGFLINYEATDASSACMEDYTENSGTFTSPNFPNNYP
NNWKCIYRITVETSQQIALHFTNFALEEAIGGQCVADFVEIRDGGYETSPPLGTYCGSIPPPRIISHSNKL
WLQFTSDFLGSGPGFSAYWDGSLTGCGGNITTPTGVFTSPSYPMPYYHSSECYWLLKASHGSPFELEFEDF
HLEHHPNCTLDYLAVYDGPSTSSHLLSQLCGNEKPPVIRSTGDSMFLKFRTDEDQQGGGFLAKYQQTCRNV
VIVNRNYGILESIHYPNPYSDNQRCNWTIQATTGNTVNYTFLAFELENHINCSTDYLELYDGPRRMGRYCG
ADMPPTGSTTGSKLQVLFYTDGVGHQEKGFQMQWFIHGCGGELSGTTGSFSSPGYPNTYPPNKECIWYITT
APGSSIQLTIHDFDVEYHARCNFDVLEVYGGPDFHSPRITQLCSQRSSENPMQVSSTGNELAIRFKTDSSI
NGRGFNASWQAVPGGCGGIFQAPNGEIHSPNYPSPYRGNTDCSWVIRVERNHRILLNFTDFDLEPQDSCIT
AYDGLSSTTTRLASVCGRQQLTNPITSSGNSLFLRFQSGPSRQGRGFRAQFNQVCGGHILTNSFDTISSPL
FPAKYPNNQNCSWVIQAQPPFNHITLSFDHFGLESSTTCTQDFLEILDGDYDDAPLRGRYCGHSMPHPITS
FSSALTLRFVSDSRVNSDGFHATYAASSSACGGTFHMAEGIFNSPGYPEVYPSNVECVWNIVSSPGNRLQL
SFITFQLEDSQDCSRDFVEVREGNATGHLVGRYCGNVLPLNYSSIVGHILWIRFVSDGSGSGTGFQATFTK
IFGNDNIVGTHGKIASPLWPGRYPHNSNYQWIVNVNATQVIHGRILEIDIEGAQSCYYDKLRVYDGLGIHS
RLIGTYCGTQTTSFSSSRNSLTFQFSSDSSITGKGFLLEWFAVNASGGPLPTIATGACGGFLRTGDAPVFL
FSPGWPESYSNSADCTWLIQAPDSTVELNILSLDIEAQRTCDYDKLVIRDGDSNLAPQLAVLCGREIPGPI
RSTGEYMFIRFTSDFSITGAGFNASFHKSCGGYLHADRGIITSPQYPETYSPNLNCSWHVLVQSGLTIAVH
FEQPFQIPSGDSSCSQGDYLVLKNGPDIYSPPLGPYGRNGHFCGSRPSSTLFTSDNQMFVQFISDGSNGGQ
GFKIKYEAKSLACGGNIYIHDVNSAGYVTSPGHPNNYPQHADCNWLIAAPPGKLIRVQFEDQFNIEETPNC
VSNYLELRDGVDSNAPLLAKLCGRSLPSSQLSSGEVMYLRFRSDNSSTQVGFKIKYAIAQCGGRVTGQSGI
IESSGYPTLPYRDNSFCEWHLKGPSGHYLTIHFEDFHLQNSSGCEKDFVEIWENHTSGNLLGRYCGNTIPD
SIDTSSNVALVRFVTDGSVTASGFRLRFESSMEACGGELQGPTGTFTSPNYPNPNPHGRVCEWRIMVQEGR
RITLTFNNLRLEAHPSCYSEHVTIFNGIRNNSPQLEKLCGSVNASSEIKSSGNTMKVVFFTDGSRPFGGFS
ATYTSSEDAVCGGSLTHFPEGNFTSPGYNGVSNYSRNLNCEWTLSNPNQGNSSIYIHFEDFYLESHQDCQF
DVLEFRVGNADGPLMWRLCGPSKPIVPLVIPYPEVWIHFVTNEHVEHVGFHAEYSFTDCGGIQLGESGVIA
SPNYPASYDSLTHCSWLLEAPQGFTITLTFSDFDIEDHATCAWDSVSVRNGGSPGSPIIGQYCGTSNPRTI
QSGSNQLVVIFNSDHSVQNGGFYATWNTQTLGCGGILHSDNGTIRSPHWPQNFPENSRCSWTVITHESKQL
EISFDNNFRIPSGDGQCQNSFVKVWAGTEEVAESLLATGCGNVAPGSILTPRNVFIAVFQSQETPAQGFSA
SFVSRCGGNFTNPSGYILSPNYPRQYDNNMNCTYIIEADPLSVVLLTFESFHLEARSAITGSCANDGVHII
RGSNLSSTPFATVCGNEILSPVTILGPVLLNFYSNAHTTDLGFKFNYKITSCGGVFNVSTGVIKSPAYSYS
DYPNNIYCLYTIVGRDDRVVQLKFSDFDVVPSTFCSQDYLAIYDGSNISDPLLGKFCGSNLPPNIKSSNHS
MLLVFKTDSFQTARGWKITFQQTLGPQQGCGGYLTGSDNTFASPDSDSNGRYDKNLNCVWFIIAPVNKLIK
LTFNTFALEAQSAMQRCIYDYVKLYDGDSENANLAGTFCGSTVPAPFISSGNFLTVQFVSDVTLEREGFNA
TYTTVDMPCGGTYNATWTPQSISSPNSSNPEVPLSMCMWFLEAPPHQQVKITVWALELHSQDCDQNYLEFR
DSPESNGSPGPQICGRNASATPTFYSSRSTAIVIFKSEVLNRNSRVGFTYQIAGCNREYNKAFGNLKSPGW
PDNYDNNLDCTVILTAPQNHTISLFFHSFGIEDSSECTHDFLEVRNGSDSSSPLFGTYCGTLLPDPIFSRN
NKLYLRFKTDSATSNRGYEIVWTSSPSGCGGTLYGDSGSFTSPGYPGTYPNNTDCEWAIIAPAGRPVTVTF
YFISIDDPGDCVQNYLILYDGPDANSPSFGPYCGADTNIAPFVASSHRVFIKFHAEYAVYPSAIRLTWDS.

domains and 27 CUB domains, is the same as seen in human and rat. All of the other structural features of human and rat cubilin were conserved in the dog protein, including a furin cleavage site after Arg32, N-terminal heptad repeats recently suggested to mediate trimer formation, 154 Cys residues involved in the 77 disulfide bridges of 8 EGF and 27 CUB domains, and the first two cysteines were missing in CUB domain 13. The only cysteine outside of the CUB domains and EGF repeats is located in the 110-amino acid N-terminal sequence. Six peptides derived by protein sequencing of purified canine cubilin were found to match identically the deduced amino acid sequence. Three peptides derived by endoLys-C digestion, KIKLNEEDLGEXLHQ, IDFQQPRMATERG, and KLVDLERK, were all in the first 120 amino acids N-terminal to EGF domain 1. Three tryptic peptides, PFYPNVYPGER, VTGQSGIIESSGYPT, VGNADGPLMXR were in CUB domains 4, 17, and 19, respectively. These results confirmed the clones under study correspond to canine kidney cubilin cDNA.

Discussion

At the start of this porject, no sequence information on canine cubilin (IFCR) was available except those 6 peptides derived from the amino acid sequencing of purified canine kidney cubilin. We began with immunoscreening by using polyclonal rabbit anti-dog cubilin antibody derived from the immunization of rabbits with IF-Cbl affinity column purified canine renal cubilin (Fyfe et al., 1991a). The cDNA library used for screening was constructed in the Lambda ZAP II® expression vector, and protein expression can be induced by isopropylthio-β-D-galactoside (IPTG). Nylon membranes presoaked with IPTG were used to lift the phage plaques and induce the expression of inserts. Then the membranes were screened with rabbit anti-dog cubilin antibody and sheep anti-rabbit IgG antibody alkaline phosphotase conjugate sequentially (Broome and Gilber, 1978). After four rounds of screening, positive clones were selected and excised into pBluescript phagemids and characterized.

Totally, 12 individual clones were purified and sequenced. Sequencing results showed that they corresponded to 8 different genes. Using these sequencing to do Genbank database searches, three matched with a previously characterized genes, such as mitosin, novel GTPase Rab 28 and HIV "TATA" element modulatory factor. Four of them matched anonymous genes with different degrees of similarity, and one had no match to any known gene. Subsequently, alignment between the clones identified by immunoscreening and the canine cubilin cDNA clone showed no similarity of any of those genes to cubilin.

Cubilin is a giant receptor with unique and important secondary and tertiary structures which provide multiple potential binding functions. These secondary and tertiary structures are also critical for anti-dog cubilin antibody recognition since the antibody bound poorly to 2-mercaptoethanol reduced cubilin in either ileum or kidney western blots (see chapter 3 for detail). The maximum cDNA length of the inserts in our cDNA library is about 5-6 kb which is only half of the full length of cubilin. The epitopes formed by these partial protein fragments might not be proper enough to be recognized by our anti-cubilin antibody. This may be why immunoscreening of canine cubilin from that canine kidney cDNA library failed. In addition, the inserts had been randomly ligated to the vector during cDNA library construction. Library directionality is also critical for immunoscreening.

The present data provide the molecular characterization of the primary structure of canine cubilin, previously known as the intestinal receptor for IF-Cbl. The predicted canine cubilin structure showed the same unique tandem domains as human and rat cubilin, which suggests that they are critical for its functions. The 8 EGF domains at the N-terminus of canine cubilin showed the conserved EGF-like domain signature which is critical for protein-protein interactions and biological function (Blomquist et al., 1984; Rao et al., 1995). Two of the EGF domains contain the discontinuous consensus sequence for calcium-binding. Calcium depletion by EDTA can completely prevent IF-Cbl binding to cubilin which indicates that the conformational change induced by calcium binding to the EGF-like domains may be important for the IF-Cbl interacting with cubilin.

The CUB domain consists of 110 amino acids defining a characteristic hydrophobicity pattern predicted to form anti-parallel β-barrels (Bork and Beckmann, 1993). The four conserved cysteines, generally thought to form two disulfide bridges, are found in all but domain 13 of cubilin that lacks the first two cysteines. This feature can be found in all three species and suggests that this domain may have a more relaxed structure than the other CUB domains. In human, but not in dog or rat, the last two cysteines in domain 6 were also missing, which may indicate the unique structure in this region for human.

Molecular dissection of rat cubilin showed that the N-terminal cubilin region conveys membrane association, and the CUB domain region convey ligand binding (Kristiansen et al., 1999). Further dissection of active binding fragments localized the binding site for IF-Cbl to CUB domains 5-8. Two mutations identified in cubilin genes of human patients with I-GS are both related to the critical region defined for IF-Cbl binding (Aminoff et al., 1999). The FM2 mutation, suggested to activate a cryptic intronic splice site leading to truncation of the receptor, was defined in CUB domain 6. Most likely the FM2 mutation does not produce a stable cubilin protein and is functionly null. A P/L subsitution (FM1) may cause a structural change specifically impairing IF-Cbl binding and is located in CUB domain 8.

The canine cubilin cDNA we cloned in this study provided the basis for mutation identification in the affected dog cubilin gene and further characterization of cubilin protein, including *in vivo* expression of large quantities

of the protein. The full-length cubilin cDNA also provided the basis for genomic structure analysis for canine cubilin.

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CHAPTER 3

CUBILIN EXPRESSION AND POST-TRANSLATIONAL MODIFICATIONS IN THE CANINE GASTROINTESTINAL TRACT

Introduction

Cobalamin (vitamin B_{12}, cbl) is an essential micronutrient which all higher animals, including humans, must obtain from dietary sources (Kapadia, 1995). 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 (Batt et al., 1989; Simpson et al., 1993; Fyfe, 1993). Endocytosis of the IF-cbl complex is mediated by a specific receptor expressed in intermicrovillar clefts of apical membrane brush-borders on villus tip enterocytes of the distal small intestine (Hammond et al., 1994; Levine et al., 1984). 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 renal proximal tubule, yolk sac, and placental epithelia, tissues exposed to very little, if any, IF-cbl. Two lines of investigation converged recently when the receptor mediating IF-cbl endocytosis was shown to have immunologic and functional identity with gp280 (Seetharam et al., 1997), a glycoprotein known as the "target of teratogenic antibodies" and demonstrated to be a recycling plasma membrane receptor in rat yolk sac epithelium (Le Panse et al., 1997).

Cubilin cDNAs of 11.3-11.6 kb encoding a highly conserved 3620 amino acid sequence were cloned, first from rat yolk sac and kidney (Moestrup et al., 1998) and subsequently from human (Kozyraki et al., 1998) and dog (Xu et al., 1999) kidney. The encoded intrinsic factor-cobalamin binding receptor, previously

called IFCR, has been given the name cubilin in recognition of its unique protein structure, including a tandem arrangement of 27 CUB domains, and recognition that it is a multiligand receptor. In addition to IF-cbl, recently identified cubilin ligands include apolipoprotein-A1 and high-density lipoproteins (Hammad et al., 1999; Kozyraki et al., 1999), albumin (Birn et al., in press), and immunoglobulin light chains (Batuman et al., 1998). Northern and western blots of rat tissues demonstrated cubilin expression in kidney, small intestine, and yolk sac, but not in liver (Moestrup et al., 1998). Renal cubilin appears to be a peripheral membrane protein because it lacks a transmembrane domain or glycophospholipid 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 (Seetharam et al., 1981b). 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 (Birn et al., 1997; Moestrup et al., 1998, Hammad et al., 2000). The size of detergent solubilized and affinity purified canine intestinal IFCR has been determined variously as 180 to 230 kDa by gel filtration, SDS-PAGE, or amino acid analysis (Seetharam et al., 1981a; Seetharam et al., 1982). In contrast, the cDNA of canine renal cubilin suggests a molecular mass of at least 400 kDa (Xu et al., 1999). For this reason, some investigators have questioned the identity of intestinal IFCR and renal cubilin (Guéant et al., 1999).

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 to investigate regulation of intestinal cubilin expression, we have examined differential expression of cubilin along the crypt to villus axis in dog ileum and regional expression of cubilin/IFCR ligand-binding activity, immunoreactivity, and mRNA along the longitudinal axis of the canine gastrointestinal tract. The longitudinal study demonstrated 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 size of intestinal and renal cubilin are due to tissue-specific post-translational modifications.

Materials and Methods

Reagents The following were purchased from commercial sources: [^{57}Co] radiolabeled cyanocobalamin (300μCi/nmol) from Amersham International (Buckinghamshire, England); anti-rabbit IgG-agarose, anti-rabbit IgG-alkaline phosphatase conjugate, p-nitro blue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) from Sigma Immunochemicals (St. Louis, MO); disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxethane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate (CDP-*Star*™) from Roche Molecular Biochemicals (Indianapolis, IN); TRIzol® reagent from Life Technologies/GibcoBRL (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-[^{57}Co]cbl complexes as described (34).

Animals and tissue isolation Animal husbandry was provided by MSU University Laboratory Animal Resources, and animal-use protocols were approved by the All-University Committee for Animal Use and Care. Twelve-20 kg body weight dogs were fasted overnight. Surgical anesthesia was induced by intravenous injection of sodium thiopental (15 mg/kg) and maintained by isoflurane inhalation. Five cm 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 ileocolic 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 snap frozen. After surgery, each dog was euthanized, without recovery from anesthesia, by intravenous overdose of pentobarbital sodium (85 mg/kg body wt).

Isolation of dog intestinal epithelial cells The methods for intestinal epithelial cell isolation used in this study were modifications of the method described by Weiser (Weiser et al., 1973). Fifteen cm-long dog ileum sections were resected starting at 40 cm proximal to the ileocecal junction and were immediately rinsed with 37°C phosphate buffered saline, pH 7.4, containing 40 mM EDTA and 2 mM DTT. The intestinal segments were filled with the same buffer, clamped at each end, and incubated with shaking for 10 min in a bath of PBS at 37°C. The lumenal solutions were collected and refilled at intervals of 10 min. After 3 fractions, the DTT concentration in the PBS buffer was reduced to 0.5 mM and 7 more fractions were collected. The collected cell suspension of each fraction was aliquoted into two tubes and a cells were pelleted. The cells in one of the tubes were used for protein and alkaline phophatase assays. The cells were washed with the PBS buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM benzamidine and frozen as cell pellet. Total protein was determined by the method of Lowry (Lowry et al., 1951), and the alkaline phosphatase activity was

determined at pH 9.2 using p-nitrophenyl phosphate as substrate (Garen A. and Levinthal C. 1960). The other aliquot of cells was used for RNA assay. They were washed with PBS buffer and lysed in the Trizol reagent (GibCo) immediately.

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 150 mM NaCl (TBS), 5 mM KCl, protease inhibitor cocktail (2 mM each of benzamidine and N-ethyl maleimide, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and 7.5 mM NaN_3. Total protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Ca^{2+}-specific receptor binding of a saturating amount (370 fmol) of IF-[^{57}Co]cbl was determined in aliquots of mucosal homogenates containing 1.25-2.5 mg of total protein by a rapid microcentrifuge method essentially as described (Hagedorn and Alpers, 1977). Under these conditions, binding was never greater than 95 fmol (25% of added ligand), and both free and bound ligand were measured after the incubation period.

Immunoprecipitation and western analysis Cubilin from canine renal cortex was used to produce anti-cubilin antiserum for 2 reasons: 1) cubilin is far more abundant in renal cortex than in ileal mucosa (Fyfe et al., 1991), and 2) in order to examine the cross immunoreactivity of canine ileal and renal cubilin. Canine renal cubilin was affinity purified to homogeneity as previously described (Fyfe et

al., 1991), and rabbits were immunized by subcutaneous injection of the protein in oil/water emulsion. Rabbit serum was harvested, and anti-cubilin activity was characterized. Ten μL 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 ethylenediaminetetraacetic acid (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 were then centrifuged at 20,000 x 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 then centrifuged at 12,000 x g for 2 min to remove nonspecific binding proteins. Cubilin was immunoprecipitated from the precleared supernatants by sequential addition of 20 μL anti-canine cubilin rabbit serum and 30 μL anti-rabbit IgG agarose beads, again with 4 h incubation at 4°C after addition of each reagent. The protein-agarose bead complexes were washed 3 times in TBS containing 0.4 % Na deoxycholate and 1 mM PMSF and boiled in 20 μL non-reducing 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-canine 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 by incubating membranes in NBT/BCIP. In preliminary re-immunoprecipitation experiments, it was determined that the homogenate supernatants were entirely immunodepleted of detectable cubilin by the method described above. Proteins from renal cortex homogenates were detected on western blots as above, but without prior immunoprecipitation.

Northern analysis Frozen tissue samples and cell fractionation pellets were homogenized in TRIzol® reagent and RNA was isolated according to the manufacturer's protocol. Thirty μg of total RNA from each sample were electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon membranes by standard protocols (Sambrook et al., 1989). A loading control probe of canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was produced by reverse transcription coupled polymerase chain reaction (RT-PCR) of canine testicular RNA as previously described (Fyfe et al., 1999). Similarly, an endogenous ~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'-GGCTCCTACATCTGTAAGTGTGC-3' and 5'-CACAGACTTGTTGGTTCCATCC-3', corresponding to sequences at nucleotide

positions 9618-9640 and 10157-10178 of the human megalin cDNA sequence, respectively (GenBank accession no. U33837). The PCR product was confirmed to be megalin cDNA by sequencing and demonstrated 88 % identity with the corresponding portion of the human megalin cDNA. The crypt specific control probe was derived from RT-PCR of dog ileal RNA by primers, F5'-CAAGCCAGTGTGAGCAGGAGCTA-3' and R5'-AAGCGGTTCAGGAA-GTGGAAGG-3', designed from the conserved region of mouse CRP-ductin and human DMBT1. 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 (Sambrook et al., 1989). 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, Inc, 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 x g for 1 hr. The resulting supernatant was again centrifuged at 150,000 x g for 2 hr, 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 vol 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 sec bursts. An aliquot was centrifuged at 4°C at 40,000 x g for 1 h. The pellet comprising total membranes was rehomogenized in TBS with 1 mM PMSF and 2 mM benzamidine (TBSPI). The supernatant was made 10 mM Tris-HCl, 140 mM NaCl, and 1 mM PMSF by addition of concentrated solutions of each. The remaining 10% TMB homogenate of ileal mucosa was diluted to 1 % by addition of 9 vol of cold TMB and homogenized again. The homogenate was made 10 mM $CaCl_2$ by addition of 0.01 vol of 1 M $CaCl_2$, stirred, incubated on ice for 10 min, and centrifuged at 2,500 x g for 15 min. The membrane pellet was homogenized in 9 vol of cold TBSPI, and the supernatant was recentrifuged at 16,000 x g for 40 min. The resulting brush-border enriched pellet was homogenized with 10 strokes in a prechilled Potter-Elvehjem in 4 vol 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 (Fyfe et al., 1991).

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 x g for 1 h, and the supernatants were made 5 mM $CaCl_2$. 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 (Fyfe et al., 1991). The proteins were eluted from the columns in TBS, pH 5.0, containing 5 mM EDTA and 10 mM CHAPS. $CaCl_2$ 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 CDP-*Star*™, a chemiluminescent alkaline phosphatase substrate, 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 (Fyfe et al., 1991), 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 prior to 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 ileocolic junctions. Because phosphoimager 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. In order to compare specific IF-cbl binding activity and cubilin mRNA expression, the IF-cbl binding data from each sample site were expressed similarly in figure 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 3.1) using a 1 kb portion of the canine cubilin cDNA cloned from renal proximal tubule cells as probe (Xu et al., 1999). Specific hybridization of the cubilin probe was detected in ileum but not in any other portion of 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 (Moestrup et al., 1998), 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.

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 5 dogs is shown in Fig 3.2A. 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 4

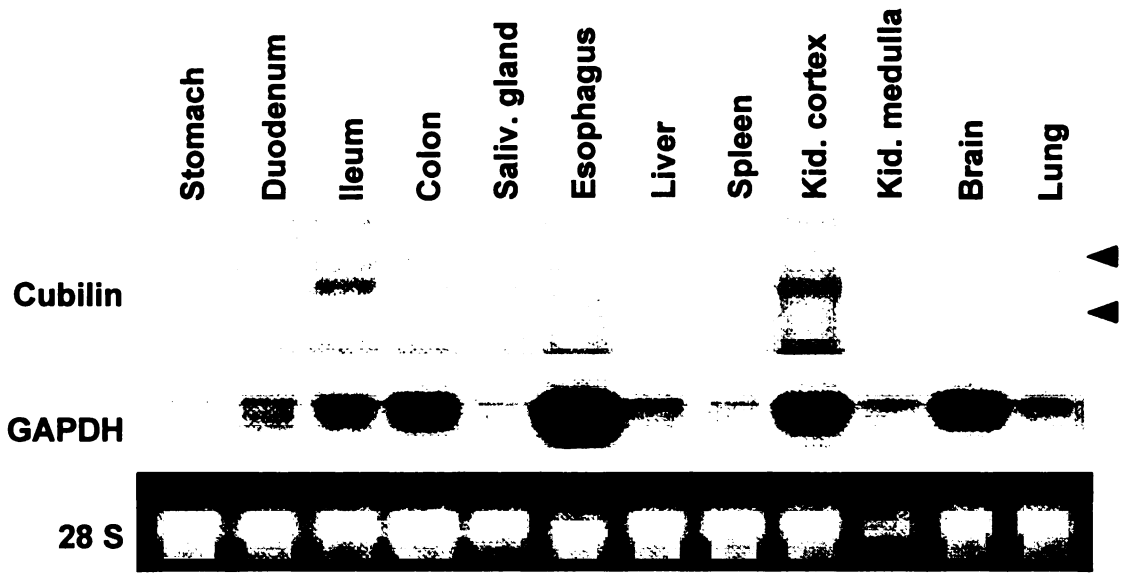
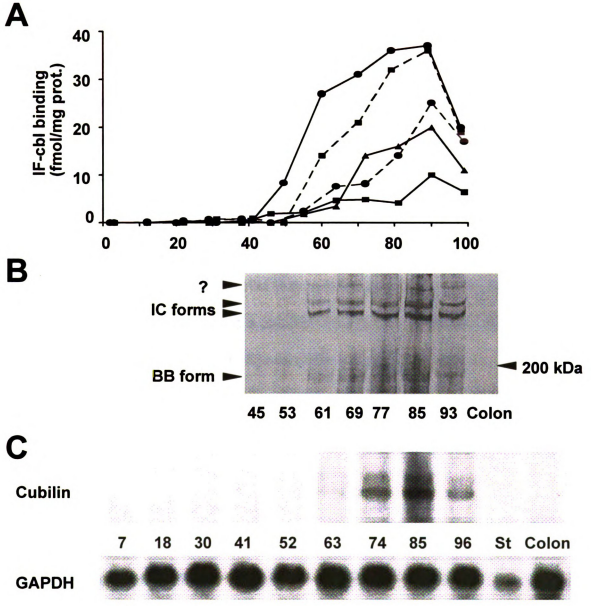


Figure 3.1: **Tissue specificity of cubilin mRNA expression.** Thirty μg of total RNA from each of several canine tissues were electrophoresed and blotted. Membranes were hybridized simultaneously to cubilin and GAPDH cDNA probes. Equality of loading was determined by examination of the 28 S ribosomal RNA on the ethidium bromide stained gel. The ~15 kb position (top arrow) was determined from subsequent hybridization to a canine megalin (gp330) cDNA probe. The lower arrow represents a 9.5 kb marker.

Figure 3.2: **Codistribution of intestinal cubilin function, immunoreactive proteins, and mRNA.** The data in panels A, B and C are aligned vertically. In each panel, the mucosal sampling site is designated by the percentage that the distance from pyloroduodenal junction to sampling site was of the full small intestinal length. **Panel A.** Mucosal IF-cbl binding specific activity was determined in intestinal segments of 5 dogs. **Panel B.** Cubilin proteins were immunoprecipitated from mucosal homogenates, separated by SDS-PAGE, and detected on blots with anti-renal cubilin antibody. The western blot of a representative dog is shown. IC indicates cubilin determined to be intracellular forms at different stages of oligosaccharide processing, and BB indicates a proteolytically modified brush-border form. **Panel C.** Thirty μg of total RNA from each mucosal segment were electrophoresed, blotted and hybridized simultaneously to cubilin and GAPDH cDNA probes. The northern blot of a representative dog is shown. *St* indicates stomach mucosal RNA.


A
IF-cbl binding (fmol/mg prot.)
40
30
20
10
0
0
20
40
60
80
100
B
?
IC forms
BB form
200 kDa
45 53 61 69 77 85 93 Colon
C
Cubilin
7 18 30 41 52 63 74 85 96 St Colon
GAPDH

other dogs, IF-cbl binding activity of mucosa overlying distal small intestinal Peyer's patches (24 ± 6.6 fmol/mg protein) was compared to 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 which paralleled the observed pattern of ligand-binding (Fig 3.2B). Four immunoreactive proteins were observed on nonreducing gels in mucosa of distal small intestinal segments, but none were observed in proximal small intestinal, stomach, or colonic mucosa. There was a band observed just under 200 kDa (labeled BB form) and 2 bands observed >400 kDa (labeled IC forms). The band observed at high apparent M_r (labeled ?) was also observed in canine renal cortex (Fig 3.4, lanes 4 and 5). None of these 4 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 3.2C). As demonstrated in Fig 3.1, no hybridizing *CUBN* transcripts were observed in stomach (St) 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.

In order 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 3.3). In the proximal to distal direction, IF-cbl binding (upper panel) and cubilin mRNA (lower panel) 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 5 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 (R^2 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.

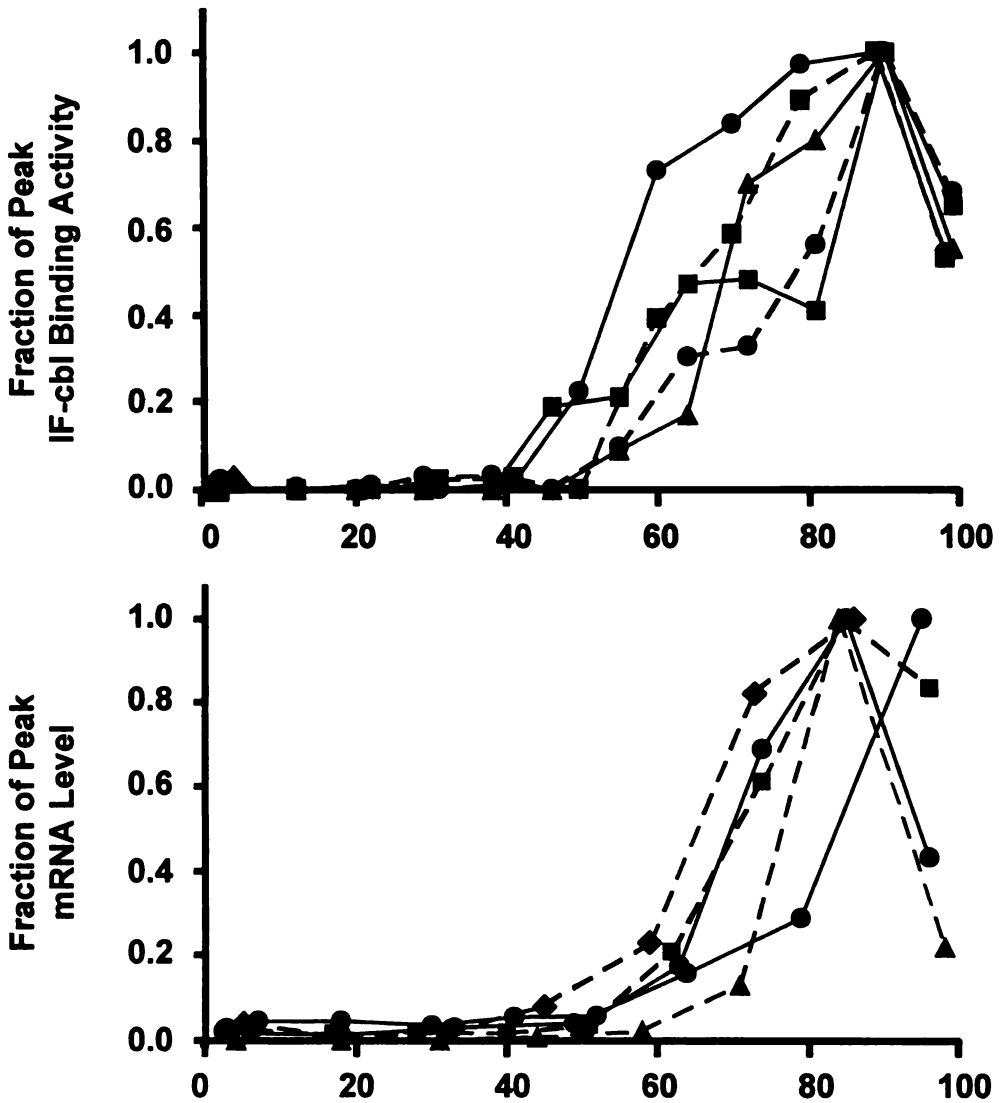


Figure 3.3: Codistribution of quantitated intestinal cubilin ligand-binding activity and mRNA. Top panel. The IF-cbl binding specific activities represented in Fig 3.2A, were recalculated as fractions of the peak value in the intestine of each dog. Linear regression of the data between the first nonzero values and the peak values of all 5 dogs had a correlation coefficient of 0.82. **Bottom panel** Relative cubilin mRNA hybridization in each mucosal sample was determined by exposure of northern blots to a storage phosphor screen. The data are plotted as fractions of the peak value found in each dog. As in the top panel, linear regression of the data between the first nonzero values and the peak values of all 5 dogs had a correlation coefficient of 0.78.

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 (Moestrup et al., 1998), 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 x 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 x g pellet (Kessler et al., 1978). 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. In contrast, enrichment of alkaline phosphatase (ALP) specific activity, a tightly associated apical brush-border membrane marker, was ~ 8.5 fold and recovery was ~ 45 % in the brush-border fraction. This suggested that some portion of cubilin may have been redistributed from the membrane to soluble fractions.

In order to examine this more closely, IF-cbl binding proteins in each of the 40,000 x g soluble, total membrane, and brush-border-enriched fractions were recovered by Triton-X-100 solubilization and affinity chromatography. They were detected on western blots of nonreducing SDS-PAGE gels with polyclonal

rabbit anti-renal cubilin serum (Fig 3.4). Cubilin from the 40,000 x g supernatant and total membrane fractions are shown in lanes 1 and 2, respectively, and cubilin recovered from the brush-border membrane enriched fraction is shown in lane 3. The IF-cbl binding proteins recovered in parallel from canine kidney cortex homogenate are shown on the same gel in lane 4. The same single major band was detected in lane 5 by western blot of canine kidney homogenate without prior fractionation or affinity chromatography, indicating that the anti-cubilin serum used as primary antibody was also immunospecific in whole tissue homogenates.

Three major cubilin bands were found in each ileal fraction (Fig 3.4). Two bands migrated just above and below the position of kidney cubilin with size estimates of 550 and 450 kDa, respectively (labeled IC), and a diffuse band migrated at ~185 kDa (labeled BB). There were also 2 faint bands of very high apparent M_r in each fraction (labeled ?). In 2 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 BB band 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 1.5-2 fold relative to the total homogenate. In addition, the smaller IC form in the brush-border fraction (lane 3) was depleted about 4 fold relative to that in the soluble fraction (lane 1) and about 2 fold relative to the total membrane fraction (lane 2). The larger IC

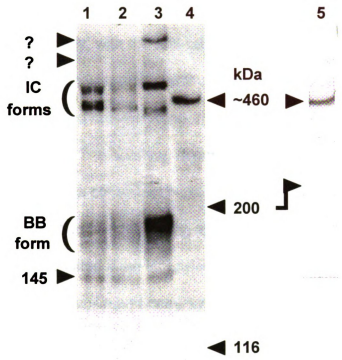


Figure 3.4: Immunoreactive and IF-cbl binding cubilin species in fractionated canine ileal mucosa and renal cortex. Cubilin species were partially purified by IF-cbl affinity chromatography from various non-detergent fractions of ileal mucosa (lanes 1-3) or renal cortex homogenate (lane 4) and separated by non-reducing SDS-PAGE on 5 % gels. Ileal mucosa fractions were 40,000 x g supernatant of total homogenate (lane 1), membranes pelleted at 40,000 x g from total homogenate (lane 2), and brush-border membranes enriched from homogenates by $CaCl_2$ precipitation (lane 3). For comparison, immunoreactive cubilin of renal cortex homogenate without IF-cbl affinity purification is also shown (lane 5). Cubilin species were detected on western blots with polyclonal anti-canine renal cubilin serum and an anti-rabbit IgG-alkaline phosphatase conjugate, either by chemiluminescent exposure of autoradiographic film (lanes 1-4) or by incubation in alkaline NBT/BCIP solution (lane 5).

form represented about 20 % of cubilin in all 3 fractions. The 145 kDa band was judged to be nonspecific crossreactivity because it was always present in the chemiluminescent detection system as the only band detected and no decrease was observed 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 [^{125}I]-protein A. Detection with the latter 2 control reagents suggested that the 145 kDa band represented crossreacting, nonreduced canine IgG from lymphocytes in ileal mucosa. The seeming enrichment of the very high apparent M_r form (?) in lane 3 was not a consistent finding and often occurred in the other fractions. Inclusion of disulfide bond reducing agents in the gel loading buffer in preliminary experiments resulted in an increased apparent M_r, but 10-20 fold reduction in signal, of renal cubilin and complete failure to detect ileal cubilin bands (Fig 3.5).

The same set of 3 cubilin bands was also recovered from the final supernatant after pelleting the brush-border fraction at 16,500 x g. In order to examine whether cubilin had redistributed to a truly soluble fraction, ileal mucosa was again homogenized without detergent, but in neutral isotonic buffer without $CaCl_2$, and centrifuged at 40,000 x g for 1 hr, and the resulting supernatant was centrifuged again at 150,000 x g for 2 hr. IF-cbl binding proteins were affinity purified from each fraction and western blotted as above. All 3 of the cubilin forms identified above were present in each of these fractions, but there was no enrichment of the BB form or diminution of the smaller IC form apparent in any fraction (Fig 3.6). Immunoreactive cubilin was roughly equally distributed

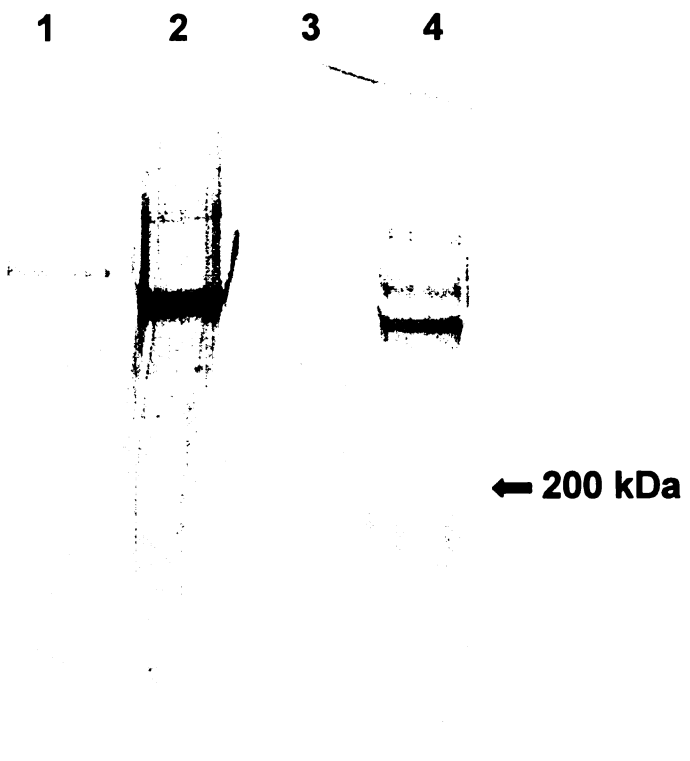


Figure 3.5: Western blot of canine cubilin in reducing and non-reducing condition. Canine cubilin purified by affinity chromatography from normal dog kidney (lanes 1 and 2) and normal dog ileum (lanes 3 and 4) was loaded with 100 mM DTT (lanes 1 and 3) and without DTT (lanes 2 and 4) onto 5% polyacrylamide gel. The proteins were detected by sequential incubation in polyclonal anti-canine renal cubilin serum, anti-rabbit IgG-alkaline phosphatase conjugate, and alkaline NBT/BCIP solution.

between the 150,000 x g supernatant and pellet fractions. Thus, none of the identified ileal cubilin forms appeared to be tightly membrane bound.

Oligosaccharide analysis

To further characterize the apparent size and tissue-specific differences of cubilin species, proteins purified from the 40,000 x g soluble fraction of ileal mucosa and renal cortex homogenates by IF-cbl affinity chromatography were subjected to differential glycosidase digestion prior to nonreducing electrophoretic separation and western blotting (Fig 3.7). 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 digestions 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 cobalamin malabsorption due to failure of brush-border cubilin expression (Fyfe et al., 1991). There was nonspecific protein degradation apparent in both mock and real digestions of renal cubilin that was not apparent in digestions of ileal cubilin. Endo H digestion of renal cubilin shifted its migration only slightly, whereas PNGase F digestion reduced the apparent size to about 400 kDa. Endo H digestion of ileal proteins reduced the apparent size of the ~450 kDa band to ~400 kDa but had no affect 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 bands to ~400 kDa and the ~185 BB form to 145 kDa. As previously described (Fyfe et al., 1991), the BB form was

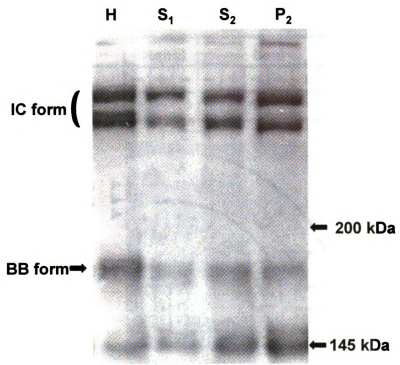


Figure 3.6: Distribution of immunoreactive cubilin forms in different centrifugation fractions of dog ileal homogenate. Ileal mucosa was homogenized without detergent or $CaCl_2$ (lane H), and centrifuged at 40,000 x g for 1 hr. The resulting supernatant (lane S_1) was centrifuged again at 150,000 x g for 2 hr to produce a final supernatant (lane S_2) and membrane pellet (lane p_2) IF-cbl binding proteins were affinity purified from each fraction and western blotted as described for Fig 3.4.

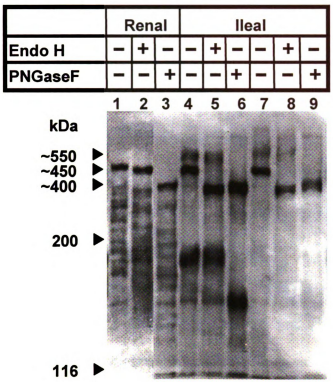


Figure 3.7: Oligosaccharide analysis of affinity purified ileal and renal cubilin. Renal cubilin of a normal dog (lane 1-3), ileal cubilin of a normal dog (lanes 4-6), and ileal cubilin of a dog affected with an inherited defect blocking brush-border expression of cubilin (lanes 7-9) were concentrated on IF-cbl-agarose beads. Eluted proteins were dialyzed and mock digested (lanes 1, 4, and 7), digested with endo H (lanes 2, 5, and 8), or digested with PNGase F (lanes 3, 6, and 9) prior to separation by non-reducing SDS-PAGE on 5 % gels. Proteins were detected by chemiluminescent western blot as described for Fig 3.4.

missing from affected dog ileal cubilin species, but the remaining species exhibited similar shifts in size as normal dog ileal cubilin after both glycosidase digestions.

Cubilin expression along crypt-villus axis in dog ileum

Cell fractions isolated from ileum during sequential intervals of incubation in EDTA represent cell populations of various stages of differentiation along the crypt to villus axis. The cell populations in early fractions contain mainly apical villus cells at a late stage of differentiation. With longer incubation, the cell populations encompass the villus base and crypt cells in early stages of differentiation (Weiser et al., 1973). Northern blot analysis of these sequential cell fractions, from two dogs, showed that the cubilin transcripts could be only detected in the first 4-5 fractions with the highest level expression occurring in the first or second fractions. In these two fractions, the majority of the cells were assumed to be villus tip cells. The expression levels gradually decreased after the fourth fraction. No cubilin transcript could be detected in the last 4 fractions. GAPDH control mRNA could be detected in all cell fractions (Figure 3.8).

To confirm the reliability of the method of cell fractionation, the cell fractions from the same two dogs were tested for villus tip and crypt specific markers. Alkaline phosphatase (ALP) which is only expressed in the mature villus cells was used as a tip specific marker (Traber et al., 1992), and the canine homolog of mouse cryptidin related protein-ductin (CRP-ductin), which is only

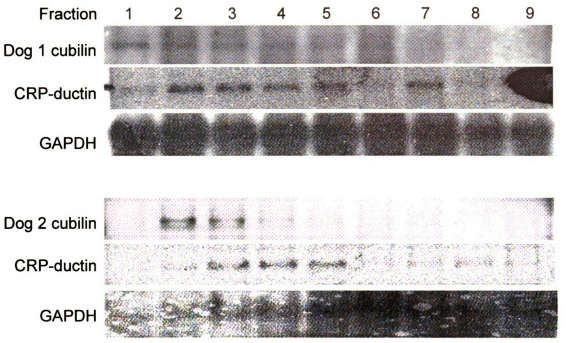
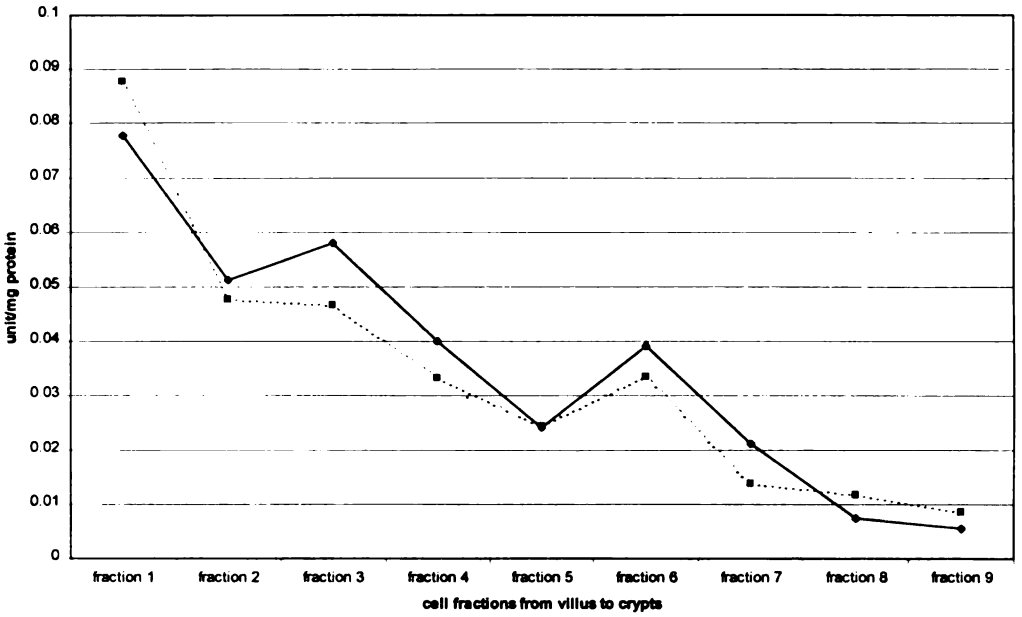


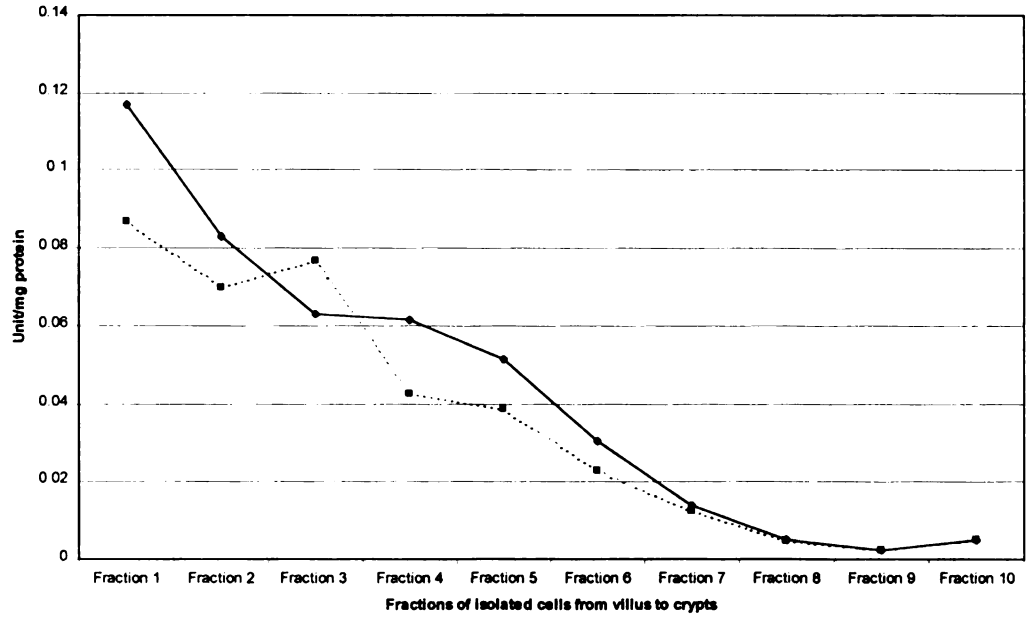
Figure 3.8: Northern blot analysis of canine cubilin and CRP-ductin along the crypt-villus axis in dog ileum. Thirty μg of total RNA isolated from dog ileal epithelial cell fractions were used for the northern blots and hybridized sequentially to dog cubilin cDNA, dog CRP-ductin cDNA amplified by RT-PCR, and loading control probe GAPDH.

Figure 3.9: Alkaline phosphotase activity along the crypt-villus axis in dog ileum. Total homogenate of dog ileal epithelial cell fractions were used for alkaline phosphotase (ALP) activity analysis after protein quantitation. The horizontal axis represents the cell fractions from villus to crypt. The vertical axis represents the ALP activity in U/mg protein. The solid and dashed line represent the duplicate experiment for the same dog.

ALP activity in ileal epithelail cells of dog1



ALP activity in ileal epithelial cells of dog 2



expressed in the immature crypt cells, was used as a crypt specific marker (Cheng et al., 1996). For ALP activity in each dog (Figure 3.9), the peak ALP activity was detected in the first fraction, and the activity decreased gradually in the subsequent fractions. These results suggested that the cell populations in the first three fractions represented mainly the villus tip cells, that cells in the middle three fractions represented a mixture of villus and crypt cells, and that cells in the last three fractions were mainly crypt cells. However, the same membrane rehybridized with the crypt specific probe (CRP-ductin) showed that the canine CRP-ductin transcript could be detected in almost all the fractions rather than just the last 4-5 fractions. High level expression of CRP-ductin was detected in fractions 2-3 for one dog and in fractions 3-5 for the other dog (Figure 3.8). These data did not support our assumption that the fractions with cubilin expression contained mainly villus tip cells.

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 observed changes in cubilin ligand-binding activity and immunodetectable protein, 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 (Baker et al., 1958; Drapanas et al., 1963; Fleming et al., 1962; Marcoullis et al., 1981) and is similar to that demonstrated by mucosal ligand binding measurements in humans (Hagedorn and Alpers, 1977). 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 receptor translocation to the plasma membrane, as has been described for glucose transporters (Kahn, 1992), or by other post-translational 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 11-12 kb transcript corresponds in size to the length of cloned renal *CUBN* cDNA (Xu et al., 1999) 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 (Xu et al., 1999), thus eliminating the possibility that we observed hybridization to multiple closely related transcripts of different genes. An alternative explanation which has not been examined is that the *CUBN* transcript may be alternatively spliced.

The polyclonal antibody used in this study was raised against purified renal cubilin of a single apparent M_r but specifically detected 3 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 co-immunoprecipitation or IF-cbl binding of one member of the complex. In preliminary experiments, it was determined that some form of receptor concentration, either immunoprecipitation or affinity chromatography, was necessary for reliable immunodetection of ileal cubilin on western blots. This may 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 (Eaton et al., 1998). The very high apparent M_r bands observed on western blots of ileal and renal cortex (labeled ? in Fig 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 a western blotting artifact. The aggregate forms in ileal mucosa were higher apparent M_r than in renal cortex (Fig 3.4), and they shifted migration on SDS-PAGE after glycosidase digestions (Fig 3.7). Both observations were consistent with the more extensive N-linked glycosylation on 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 3.7). Heavy glycosylation of ileal cubilin may be an adaptation conferring some protection from lumenal 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 (Birn et al., 1997; Kristiansen et al., 1999; Lindblom et al., 1999; Seetharam et al., 1981a; Seetharam et al., 1982). 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 monomer after disulfide bond reduction (Lindblom et al., 1999). The N-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 (Kristiansen et al., 1999). In the latter study, aggregate forms were observed on nonreducing gels only in the constructs expressing the membrane

binding N-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.

We concluded that the ~185 kDa band, labeled BB in Fig 3.2B and 3.4, is a brush-border form of cubilin based on 1) its absence from ileal mucosa of dogs exhibiting an inherited defect that blocks brush-border cubilin expression and 2) its enrichment in fractions enriched for brush-border membranes. It is likely that the ~185 kDa form arises by proteolytic cleavage of the ~550 kDa form (see below) when exposed to proteases in the intestinal lumen. This idea is consistent with the previous finding 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 (Seetharam et al., 1982). Enrichment and recovery of 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 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 with endogenous cubilin in rabbit kidney (Moestrup et al., 1998) and canine ileal cubilin reconstituted in artificial liposomes (Seetharam et al., 1981b). These are *in vitro* behaviors consistent with the designation of cubilin as a peripheral membrane protein.

What we have designated IC forms in Fig 3.2B and 3.4 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 high-mannose and derived from cubilin which has not been transported from the RER to the cis-Golgi. Furthermore, the 450 kDa band was found largely in the 40,000 x g soluble fraction after homogenization in TMB without detergents 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 they are complex or hybrid types that had been exposed to Golgi processing enzymes. The same was true of oligosaccharides on the 185 kDa form. We have not determined which of these forms is directly responsible for IF-cbl endocytosis *in vivo*. The 185 kDa and 550 kDa forms may coexist for some time on the brush-border, and it is possible that the endocytic function of cubilin is inactivated by a proteolytic cleavage to 185 kDa.

Detection of the ~450 kDa form was evidence that ileal cubilin gained IF-cbl binding activity and immunoreactivity at a step in biosynthesis prior to modification of high mannose oligosaccharides to endo H resistant forms and prior to expression in a position on the brush-border appropriate to its endocytic function. Previous data indicated that the same was true of renal cubilin (Fyfe et al., 1991). 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 cells that express cubilin are largely a population of fully differentiated cells whereas ileal enterocytes that express cubilin in a homogenate of mucosa are a mixture of proliferating and immature crypt cells, partially differentiated mid-villus cells, and fully differentiated villus tip cells. Thus, in contrast to kidney, homogenates of ileal mucosa are relatively enriched in cubilin forms produced early in the biosynthetic pathway and the mature forms are underrepresented.

Removal of N-linked oligosaccharides from both IC forms of ileal cubilin resulted in migration at ~400 kDa, consistent with the polypeptide molecular weight deduced from the canine renal cubilin cDNA (Xu et al., 1999). Similar to most glycoproteins destined for the plasma membrane, it appears that canine ileal cubilin is translated as a 400 kDa polypeptide, that it is N-linked glycosylated in the RER to ~450 kDa, and thereafter, that oligosaccharide modifications produce a mature ~550 kDa glycoprotein. However, as shown in Fig 3.7, dogs exhibiting an inherited defect blocking brush-border expression of cubilin still produce both IC forms of cubilin in the intestine, including the endo H resistant, ~550 kDa form. Genetic linkage evidence indicates that the disease locus in these dogs is not the cubilin locus (Xu et al., 1999), and therefore, some type of cubilin-specific transport to the lumenal brush-border surface must exist. The present data suggest that it operates after cubilin matures to the endo H resistant, ~550 kDa form in ileal mucosa, but a study in rat yolk sac cells suggests that newly synthesized cubilin arrives on the brush-border with endo H

sensitive oligosaccharides (Baricault et al., 1995). It is possible that these phenomena are tissue or species specific.

Tissue-specific differences of N-linked oligosaccharides may also have contributed to confusion in the literature about ileal cubilin protein size and lingering thought that intestinal and renal cubilin ligand-binding activity might be due to expression of different but closely related genes (Guéant et al., 1999). The present data indicated that ileal and renal cubilin in dogs have the same protein backbone and that differences of apparent M_r on SDS-PAGE are due to differing N-linked glycosylation. 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 cobalamin (Baker et al., 1958; Drapanas et al., 1963; Fleming et al., 1962; Marcoullis and Rothenberg, 1981). Conversely, antibody raised against purified canine ileal cubilin demonstrated cross immunoreactivity with canine renal cubilin in a previous study (Fyfe et al., 1991). However, recent reports of a null mutation in the cubilin gene of a human individual exhibiting both intestinal IF-cbl malabsorption and urinary loss of cubilin ligands provide the most elegant and conclusive demonstration that ileal and renal cubilin functions are expressions of the same gene (Aminoff et al., 1999; Kozyraki et al., 1999). The findings reported here will aid in determining the basis of the analogous disorder in dogs.

Previous immunoelectron microscopic study (Levine et al., 1984) of intracellular cubilin distribution during enterocyte maturation from crypt to villus

tip of ileal mucosa showed that synthesis of cubilin protein could be easily detected in the mid-villus cells. The expression level of cubilin dramatically increased in the villus tips, and expression of cubilin was extremely low in the crypt cells. Northern blot of canine cubilin mRNA expression in this study showed that cubilin transcripts could only be detected in the early cell fractions assumed to represent villus cell populations. These results were supported by the parallel ALP activity which is known to be expressed only in villus cells. However, the northern blot of crypt specific canine CRP-ductin showed a conflicting result. CRP-ductin was isolated from cDNA libraries of mouse intestinal crypt cells (Cheng et al., 1996). *In situ* hybridization in the same study showed that in the small intestine, CRP-ductin mRNA is expressed in crypt cells at all stages of differentiation from the stem cells to the terminally differentiating cells of the crypt top, but not in the mature cells of the villus. A recent study (Takito et al., 1999) suggested that mouse CRP-ductin, human DMBT1, rabbit hensin and ebnerin are the products of alternative splicing of a single gene. The transcript of this gene for all four family members could be detected in the small intestine, but the crypt specificity has only been determined in mouse.

The conflicting results for the ALP activity and the canine CRP-ductin expression may have been due to incomplete cell fractionation. The incubation condition may have been over-extensive which caused both villus and crypt cells to fall-off in the early fractions. The crypt specificity of canine CRP-ductin also needs to be confirmed. To eliminate all these concerns, other crypt specific markers could be used to determine the origin of cell population in each fraction,

such as thymidine kinase. Alternatively, *in situ* hybridization of canine cubilin directly on the dog ileum sections could be used to determine the cubilin mRNA expression along the crypt to villus axis.

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CHAPTER 4

LINKAGE ANALYSIS OF CANINE SELECTIVE COBALAMIN MALABSORPTION WITH *CUBN* AND MEGALIN LOCI

Introduction

Hereditary megaloblastic anemia 1, (*MGA 1*, OMIM #261100), also known as Imerslund-Gräsbeck syndrome (IG-S), is a rare autosomal recessive disorder characterized by selective intestinal cobalamin (vitamin B_{12}) malabsorption leading to juvenile-onset severe megaloblastic anemia and/or neurologic manifestations. Most patients also exhibit mild proteinuria. The disorder has been described in families in Norway (Imerslund 1960), Finland (Gräsbeck et al., 1960), Israel (Ben-Bassat et al., 1969), Saudi Arabia (Abdelaal and Ahmed, 1991), Turkey (Altay et al., 1995; Altay and Cetin, 1999) and sporadically worldwide. *MGA 1* was mapped to chromosome 10p12.1 in Finnish, Norwegian and a Saudi Arabian kindred (Aminoff et al., 1995, 1999).

One candidate gene for IG-S was cubilin, a 460 kDa protein found in the apical membrane domain of epithelial cells of the distal small intestine, and renal proximal tubules (Seetharam et al., 1997; Birn et al., 1997). Human (GenBank accession # AF034611; Kozyraki et al., 1998) and rat (GenBank accession #AF022247; Moestrup et al., 1998) cubilin were recently cloned. The human *CUBN* locus was mapped within the *MGA 1* locus by fluorescence *in situ* hybridization, radiation hybrid mapping, and screening of YAC clones (Moestrup et al., 1998). Two mutations, a missense mutation in CUB domain 8 and an intronic point mutation activating a cryptic splice site within CUB domain 6, were recently demonstrated in Finnish kindreds (Aminoff et al., 1999).

A canine model with selective intestinal Cbl malabsorption was identified with clinical and laboratory features similar to those seen in human (Fyfe et al.,

1991a). Immunoelectron microscopy and cell fractionation studies showed failure of cubilin expression on the apical brush-border membrane of either tissue (Fyfe et al., 1991b). Characterization of the urine proteins of affected dogs showed ApoA-1 (Kozyraki et al., 1999), albumin (Birn et al., in press), haptoglobin, IgG light chains and other unidentified proteins (Fyfe et al., unpublished data), which are the same as the urine proteins identified in the patient with a cryptic splice site (null) mutation of cubilin. To further determine whether defective function of canine cubilin is caused by a mutation within its coding sequence, which is the most common reason for a protein trafficiking disorders, we perfomed the linkage analysis to determine whether the cubilin gene locus and the disease locus are genetically linked.

The predicted protein structure of human and rat cubilin showed no sequence compatible with a transmembrane domain nor a signal for glycosylphospho-tidylinositol anchor addition. Parallel with the predicted structure, the protein showed only a weak association with the plasma membrane (Moestrup et al., 1998). All these data suggested that cubilin is a peripheral membrane protein. As a peripheral membrane protein without internalization signals, cubilin-mediated endocytosis of the IF-Cbl complex may be facilitated by another membrane protein. Megalin, a transmembrane protein with an endocytic signal sequences in the cytoplasmic domain, interacts with cubilin *in vitro* (Moeatrup et al., 1998), colocalizes with cubilin *in vivo* (Sahali et al., 1988, 1993, Birn et al., 1997, Moestrup et al., 1998) and exhibits coregulation of expression (hammad et al., 2000). Megalin, therefore, has been suggested to

play an important role in facilitating the endocytosis of the IF-Cbl complex. Therefore, a defect in the interaction between cubilin and megalin may be another reason for failure of cubilin expression on apical brush border membranes. To determine whether the defective interaction between megalin and cubilin caused the Cbl malabsorption in this canine model, we also conducted linkage analysis between canine megalin and selective intestinal cobalamin malabsorption in this dog family.

Materials and Methods

Cubilin linkage study

Dogs in the linkage studies were members of a breeding colony maintained at Michigan State University; protocols for husbandry and tissue collection were approved by the MSU All-University Committee for Animal Use and Care. The parents and 23 offspring, including 13 affected and 10 clinically-normal carriers, of matings between an affected female and an obligate carrier male were studied. The disease phenotype of each dog was determined by the following criteria: affected dogs became inappetent and failed to gain weight after 10-14 weeks of age; they had low serum cobalamin concentrations, methylmalonic aciduria, mild nonregenerative anemia, and neutropenia. And all hemotologic, metabolic, and growth abnormalities were reversed by parenteral cobalamin administration. Due to the carrier X affected matings strategy, offspring determined as not affected were considered obligate carriers of selective cobalamin malabsorption.

Genomic DNA of normal dogs was isolated from frozen liver samples. Approximately 0.5 g of frozen tissue from each dog was ground into fine power in liquid nitrogen and gently spread onto extraction buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 20 μg/ml pancreatic RNAase, 0.5% SDS), allowing the powder to become submerged. Tissue suspensions were incubated with 100 μg/ml proteinase K at 55°C overnight and were extracted in 0.5 M Tris-HCl (pH 8.0) equilibrated phenol, phenol and chloroform mixture, and chloroform sequentially and precipitated by 10 M ammonium acetate (0.2 vol/vol) and 100% ethanol (2

vol/vol). Isolated DNA was washed in 70% ethanol and resuspended in TE pH 8.0, and quantified by spectrophotometry.

Genomic DNA was amplified by PCR using various combinations of the primers 838F, 5'-AGCCTGCGTGCTGGACATCGAC-3'; 947F, 5'-GGATGGCAAGGAAATGGATATAGT-3'; 1150R, 5'-TGGGTGGCAGCCTC-CATTATTGA-3'; and 1341R 5'-CCAGCCCAACCTGATTCACACTTA-3' designed from the canine cubilin cDNA sequence. 50 μL reaction mixtures contained 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.4 mM each deoxynucleotide, 0.5 μM each primer, 500 ng of DNA template, and 2.5 U of *TaKaRa LA Taq* polymerase (PanVera, Corp., Madison, WI). After a hotstart denaturation at 94°C for 2 min, reactions were carried out for 35 cycles of denaturation at 98°C for 20 s and annealing / extension at 68°C for 20 min. Reaction products were gel purified and partially sequenced using the above PCR primers.

According to the sequencing results, a 0.9 kb intron of both an obligate carrier male and an affected female were amplified using primer 947F and 1150R. The PCR products were gel purified and cloned into the plasmid PCR™ II (Invitrogen®, San Diego, CA) at 16°C overnight. Recombinant plasmids were transformed into DH5 $_{\alpha}$ competent cells. Six individual clones from each dog were purified by QIAGEN plasmid purification kit and sequenced to identify intronic sequence variations.

DNA templates used for PCR amplification of a cubilin intron variation (CIV) were isolated from snap frozen liver or from fresh blood samples. The DNA

from frozen tissues was isolated by the same protocol as previously described. The DNA from whole blood cells was isolated by the following procedure. The whole blood cells were lysed by ST buffer (0.32 M sucrose 10 mM Tris, pH7.6, 5 mM $MgCl_2$ and 1% Triton X-100) and the leukocyte nuclei were resuspended in NE buffer (75mM NaCl and 25 mM EDTA pH 8.0) and 0.1% SDS. After sequential incubation with proteinase K and RNAse A, the genomic DNA was extracted and precipitated as from frozen tissue described previously. To PCR amplify the intron variation, the primers (CIVF, 5'-GATCACAGGCCTACAGCTC-CATT-3' and CIVR, 5'-CCAGGCCAACCAGAGATCTTCTA-3') were designed to flank a 17 base insertion sequence. PCR produced allele-specific amplification products of 199 and 182 bp. PCR reactions were 50 μL, containing 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 2 mM $MgCl_2$, 0.37 mM each deoxynucleotide, 0.25 μM each primer, and 100 ng of DNA template. Taq DNA polymerase (2.5 U) was added after a 5 min hotstart at 95°C, and PCR reactions were carried out for 28 cycles of denaturation for 30 s at 95°C, annealing for 60 s at 65°C, and extension for 120 s at 72°C. The reaction products were electrophoresed in TAE buffer on 4% agarose gels and visualized by ethidium bromide staining.

Southern blotting was performed on genomic DNA of carrier and affected dogs isolated from frozen liver as described above. Genomic DNA was digested by *Bam* HI, *Eco*RI, *Hind* III, *Pst* I, and *Xba* I and 5 μg of each digested DNA was electrophoresed on a 1% agarose gel. DNA was transferred to a nylon membrane, and the blot was hybridized by standard methods (Sambrook et al., 1989) to a canine cubilin cDNA probe (bp 947-1150).

Megalin linkage study:

The primers used for canine megalin DNA amplification were designed from a conserved region of human and rat megalin and human low-density lipoprotein related protein (LRP1). The primer: Meg forward, 5'-GGCTCCT-ACATCTGTAAGTGTGC-3'; Meg reverse, 5'-CACAGACTTGTTGGTTCCATCC-3' were designed from human LRP1 exon 56 and exon 60 respectively. Reverse transcribed polymerase chain reaction (RT-PCR) was performed using above primers to amplify canine megalin cDNA from total RNA of dog kidney which was isolated from snap frozen dog kidney tissue using Trizol® reagent (GibCo protocol). The PCR product was gel purified and sequenced using the same pair of primers.

The same pair of primers was used to amplify the canine genomic megalin fragment. The 50 μl reaction mixture contained 300 mM Tris-HCl (pH 8.5), 75 mM ammonium sulfate, 2.5 mM $MgCl_2$, 0.25 mM each deoxynucleotide, 0.5 μM each primer, and 300 ng of dog genomic DNA template. 2.5 U of AmpliTaq polymerase (Perkin Elmer, Foster city, CA) was added after a hotstart denaturation at 95°C for 5 min, then reactions were carried out for 32 cycles with 30s denaturation at 95°C, 30s annealing at 58°C and 2 min extension at 72°C. The single PCR product from an obligate carrier dog was gel purified and sequenced. In order to separate two PCR products (both are around 2.1 kb) from an affected dog, a new forward primer, 1501F, 5'-CATTGCCTCTCTGTCTCTGACCT-3', was designed from the middle exon and

used for PCR amplification under the same reaction conditions, but without the hotstart, to produce smaller PCR products (about 700 bp). The two smaller PCR products of the affected dog were gel purified and cloned individually in the plasmid pCR™ II (Invitrogen, San Diego, CA). Two purified plasmids containing each PCR product were sequenced separately.

The primers, 1553F, 5'-GAGCACTGTGTGGATGTCAA-3'; 2009R, 5'-CATGGAGAGTCCAGTATAGG-3' were designed to flank an intronic insertion of 68 bp defined from canine megalin sequence. PCR amplification of this canine megalin intron variation was performed on DNA isolated from frozen liver or fresh blood samples using the same methods as above. Totally 24 dog samples with confirmed phenotype were used for this study, including one normal dog, 9 carrier dogs and 14 affected dogs for selective cobalamin malabsorption. The conditions for the 50 μl PCR reaction were as same as for the canine megalin genomic DNA amplification except using 1 min extension time instead of 2 min. Allele specific PCR products were separated on a 4% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining.

Results

The intron/exon structure of the canine *CUBN* was defined in the region of EGF domains 4-7 by amplification of genomic DNA by PCR, using primer sequences from the cDNA (Figure 4.1, panel A). Sequencing of the PCR products demonstrated concensus splice donor and acceptor sequences at the points where the genomic sequence diverged from the cDNA sequence. In the 5' to 3' direction, 4 introns (2.2, 0.9, 2.0 and 0.2 kb) and 3 included exons (cDNA sequences 948-1079, 1080-1175, and 1176-1294; Gly292-Pro335, Gly336-Leu367, and Gly368-Ile407) were defined. The intron/exon boundaries did not correspond to the protein structural boundaries of the EGF domains.

A canine *CUBN* variation was determined within the 0.9 kb intron between nucleotides 1079 and 1080 of the cDNA sequence in EGF domain 5 (Panel A). The intron between PCR primers 947F and 1150 R of a heterozygous male dog (F274 Figure 4.1) was amplified, and the product was cloned. Sequencing of six individual clones demonstrated a 17 bp sequence (5'-CAGAACATTGTTTATGC-3') in three of the clones inserted 187 bp 5' of the 3' intron/exon boundary. Sequencing of 6 clones of the intron similarly amplified from a homozygous female (F284, Figure 4.1) demonstrated that she was likely ($P>0.98$) homozygous for the allele containing the 17 bp insert. Single hybridizing bands of 5-10 kb, identical in carrier and affected dog DNA, were observed on Southern blots of canine genomic DNA digested with 6 different restriction enzymes and hybridized to a probe of *CUBN* cDNA sequence between bp 947 and 1150, thus

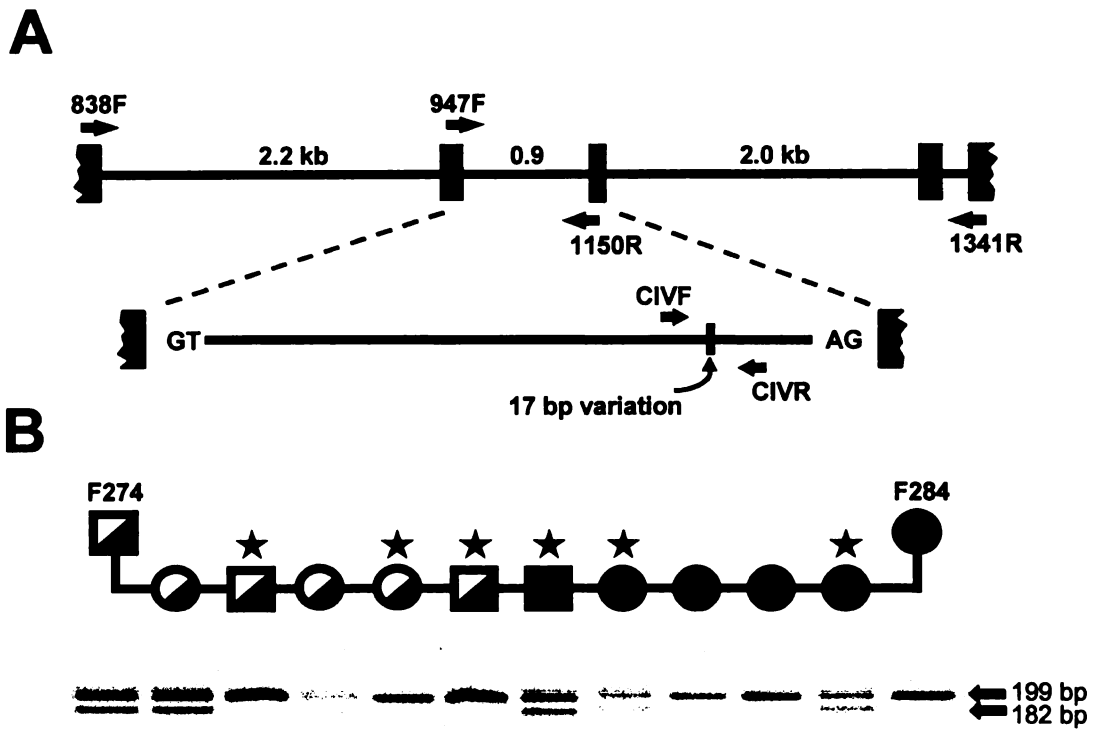


Figure 4.1: Independent segregation of canine I-GS and *CUBN* loci. (A) Four introns (horizontal lines) and included exons (vertical boxes) were defined by PCR amplification using *CUBN* cDNA primers and sequencing the products. A 17-bp variation (vertical line) was found in the 0.9-kb intron for which dog F274, an obligate carrier of canine I-GS, was heterozygous and dog F284, an affected dog, was homozygous. **(B)** Solid symbols indicate I-GS affected dogs, half-solid symbols indicate obligate carriers, squares are males, and circles are females. DNA from offspring of matings between dogs F274 and F284 was amplified by PCR using primers flanking the 17-bp variation, producing allele-specific products of 199 and 182 bp. Results of 10 offspring are shown, with the stars indicating recombinants.

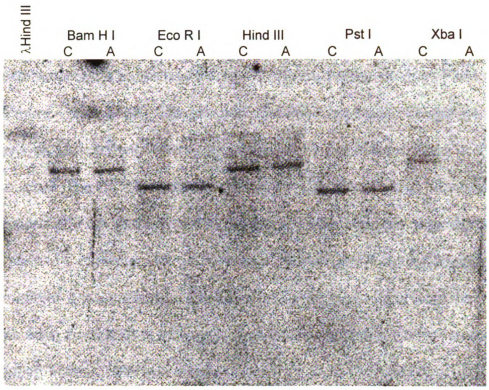


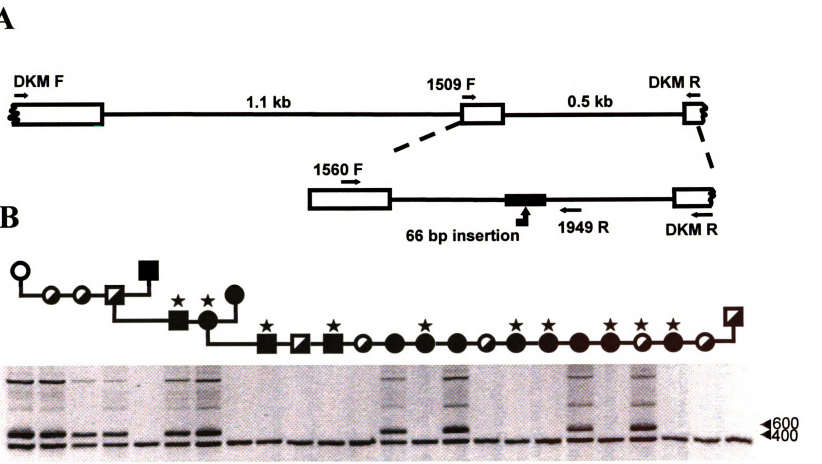
Figure 4.2: Intragenic cubilin marker is located in a unique region of the canine genome. Southern blots of canine genomic DNA digested with 6 bp recognition-site restriction endonucleases were prepared and hybridized to a canine cubilin cDNA probe flanking the intron containing an identified variation (see figure 4.1). Single hybridizing bands of 5-10 kb, identical in carrier and affected dog DNA were observed, thus confirming that the identified 17-bp marker variation was in a unique region of the canine genome. C: carrier dog, A: affected dog.

confirming that the identified 17-bp variation was in a unique region of the canine genome (Figure 4.2).

A *CUBN* allele-specific PCR test was designed to examine the 17 bp insertion variation in affected and carrier offspring of affected X carrier matings (phase-known double backcross matings) performed between dogs F284 and F274 (Figure 4.1, Panel B). The affected offspring from these matings are homozygous and clinically normal littermates which are heterozygous at the disease locus. The PCR test confirmed that the clinically affected dam was homozygous and the clinically normal, obligate carrier sire was heterozygous for the 17 bp insert. However, 13 recombinants were detected among 23 offspring of these matings, a recombination fraction (0.56) that did not differ from the recombination fraction (0.5) expected under the hypothesis of independent segregation of the *CUBN* and disease loci (X^2=0.39, df=1, P=0.53).

To test another candidate gene for this canine model with selective intestinal cobalamin malabsorption, a 2290 bp-canine megalin genomic DNA fragment was amplified by PCR using primer pairs designed from conserve regions of human and rat megalin. The alignment between dog megalin cDNA amplified from dog kidney total RNA by RT-PCR using the same pair of primers and this canine genomic DNA fragment defined the partial intron/exon structure of canine megalin. This region contained two introns (1.1 kb and 0.6 kb) and 1 included exon (156 bp) (Figure 4.3, Panel A). The canine megalin cDNA fregment showed 88% identity to human megalin, which confirmed the sequence specificity.

Figure 4.3: Independent segregatin of canine I-GS and megalin loci. (A) Two introns (horizontal lines) and included exon (box) were defined by PCR amplification using megalin cDNA primers designed from human and rat megalin conserved regions and sequencing the products. A 66-bp variation (solid box) was identified in the 0.5-kb intron and was used as internal marker for linkage analysis. **(B)** Solid symbols indicate I-GS affected dogs, half-solid symbols indicate obligate carriers, and the empty-symbol indicates a normal dog. Squares are males, and circles are females, DNA from offspring was amplified by PCR using primers flanking the 68-bp variation, producing allele-specific products of 388 and 456 bp. The results of 20 offspring showed 10 recombinants (stars).


A
DKM F
1.1 kb
1509 F
0.5 kb
DKM R
1560 F
66 bp insertion
1949 R
DKM R
B
600
400

A canine megalin intron variation was determined within the 0.5 kb intron. Subclone and sequence results of the PCR amplified 0.5 kb intron of an affected dog genomic DNA showed that one allele contained a 68 bp insertion ($AGCTGGTCTGTTTTCTT(T_{28})TGT(T_{16})TT$), but no insertion was found in the other allele. This result was in conflict with the disease locus, which is homozygous for the affected dogs. In contrast, the obligate carrier dog, which was heterozygous for the disease locus, showed homozygousity for the megalin intron variation, with no insertion in either allele. At this point, the data suggested that the disease locus was not genetically linked with the megalin locus in this family of dogs with selective intestinal Cbl malabsorption. Further family linkage study with 20 offspring (12 affected dogs and 8 carrier dogs) (Figure 4.3), using allele specific PCR amplification with primer 1553F and 2009R, producing a 390 bp product for the normal allele and a 456 bp product for the insertion allele, showed 10 recombinants (0.5 recombination fraction) and confirmed independent segregation of the disease locus and the megalin locus in this family of dogs with selective intestinal Cbl malabsorption.

Discussion

Cubilin is a biologically compelling candidate gene for selective intestinal cobalamin malabsorption with proteinuria. Cubilin is a multi-ligand receptor expressed in the small intestinal epithelium where it mediates absorption of cobalamin via binding of IF-Cbl complexes (Seetharam et al. 1997) Cubilin is also expressed in the renal proximal tubule epithelium where it mediates reabsorption of specific proteins filtered by the glomerulus (Batuman et al., 1998; Birn et al., 2000; Fyfe et al., unpublished data). *CUBN* mutations causing selective intestinal cobalamin malabsorption with proteinuria in Finnish kindreds have been defined (Aminoff et al., 1995; Kozyraki et al., 1998).

The canine model of selective intestinal cobalain malabsorption with proteinuria is a well-defined, simple autosomal recessive disorder for which *CUBN* was also an attractive candidate gene. Intrinsic factor and transcobalamin-II functions of the affected dogs were demonstrated to be normal, and immunoelectron microscopy revealed lack of cubilin in the apical membrane of distal intestinal epithelial cells (Fyfe et al., 1991a). Measurements of cubilin ligand-binding activity and western blotting of fractionated cells demonstrated that the cubilin protein was expressed in affected dog ileal and proximal tubule epithelial cells but did not reach the apical brush border membrane. Cubilin purified by detergent solubilization and affinity chromatography from normal and affected dog renal cortex comigrated on nonreducing SDS-PAGE gels but yielded different peptides upon proteolytic digestion. Additionally, Asn-linked oligosaccharides on affected dog cubilin were in an Endo H sensitive form,

suggesting retention in the endoplasmic reticulum (ER) and failure of the nascent protein to make its way through the biosynthetic pathway (Fyfe et al., 1991b).

These findings were analogous to findings in a number of disorders of secretory and membrane protein expression in which mutations of specific genes cause failure of the translation product to fold properly and to remain associated with ER chaperone proteins, to self-aggregate, or to be degraded rapidly (Kopito 1999; Bross et al., 1998; Kim and Arvan 1998). With the exception of abetalipoproteinemia (MTP, MIM #200100), all inherited ER retention/degradation disorders described to date are attributed to mutations in the coding sequence of the particular secreted or membrane protein. The similarities of the cloned canine *CUBN* cDNA sequence with those of other species and the match of cubilin peptides to the deduced amino acid sequence of the canine clone confirmed its identity (Chapter 2). Elimination of linkage of an intragenic *CUBN* marker to the inherited disorder in this canine family indicates that failure of apical membrane expression of cubilin and the resultant selective cobalamin malabsorption is caused by dysfunction of a gene product whose locus maps ≥ 50 cM from *CUBN* in the canine genome. Because cellular physiology of humans and dogs is highly homologous, these findings suggest that human I-GS may exhibit locus heterogeneity in addition to allelic heterogeneity at the *CUBN* locus. Human I-GS remains to be mapped in kindreds of various ethnic backgrounds.

Genes unlinked to the human *MGA 1* locus which may explain our findings include the gene encoding the endocytic receptor, megalin (gp330, LRP2),

mapped to 2q31 (Chowdhary et al. 1995) which has been demonstrated to associate and co-localize with cubilin (Birn et al. 1997; Moestrup et al. 1998). It has been postulated that megalin may anchor cubilin to the apical membrane and mediate its vesicular trafficking because cubilin lacks a transmembrane domain or other obvious membrane anchoring signal. Megalin, which is closely related to LRP also binds the urokinase-inhibitor complex (Nykjaer et al., 1997; Herz et al., 1992), and may perform a similar function in regard to both the urokinase receptor and cubilin-ligand complexes. The rejection of linkage between the canine megalin locus and the disease locus of this canine model with selective cobalamin malabsorption indicates that the failure of membrane expression of cubilin in affected dogs was caused by a defect of a gene other than megalin.

To locate the disease-causing gene for this canine model with selective cobalamin malabsorption, instead of genome-wide screening, linkage analysis of candidate genes were performed. Another candidate gene product is an ER chaperone, lipoprotein related protein-associated protein 1 (LRPAP 1) or RAP. RAP interacts with cubilin *in vitro* and maps to 4p16.3 (VanLeuven et al. 1995) of humans. By analogy to the function of association with megalin (LRP2) and the low density lipoprotein receptor-related protein 1/α2-macroglobulin receptor (LRP1), RAP may mediate proper folding of cubilin and prevent premature ligand binding to cubilin within the ER and later compartments of the biosynthetic pathway (Bu and Schwartz 1998). RAP is essential for the proper folding and disulfide bond formation of LRP1, findings supported by ER retention

and rapid degradation of LRP1 and other members of the LDL-receptor gene family in RAP-knockout mice (Willnow TE et al., 1996; Obermoeller et al., 1998). Future investigations of canine selective intestinal cobalamin malabsorption in this family will focus initially on the LRPAP1 or RAP locus.

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Future Direction

The major focus of future projects will be to identify the molecular mechanism of this canine model of selective intestinal cobalamin malabsorption which closely resembles the human Imerslund-Gräsbeck syndrome. In this thesis, we used linkage analysis of the disease locus with cubilin and megalin gene loci and rejected the hypothesis that cubilin or megalin was the disease causing gene product. The specific goal for future projects will be to generate new candidate genes. From this point, two approaches can be used to identify new candidate genes, which are functional analysis and positional mapping.

The rejection of genetic linkage of canine I-GS with canine cubilin and megalin gene suggested that a gene product other than megalin may physically interact with cubilin and be required in the biosynthesis or apical delivery of cubilin to the plasma membrane. Identification of proteins which interact cubilin will generate functional candidate genes.

Receptor associate protein (RAP), which interacts with cubilin *in vitro* (Moustrup et al., 1999) will be studied first. RAP is an ER chaperone-like protein (Bu et al., 1995) and may mediate proper folding of cubilin and prevent premature ligand binding to cubilin within the ER. Human and rat RAP genes are cloned and the intron/exon structure of each were identified (van Leuven et al., 1995;). An intragenic marker within the canine RAP gene can be identified by using the same approach as for megalin. Genetic linkage will be used to test linkage to the disease condition.

If the RAP gene is not linked to the disease locus, other methods, like co-immunoprecipitation, affinity chromatography, and 2-D gel electrophoresis, will be used to identify the proteins associate with cubilin. By comparing the different pattern between normal and affected dogs, association aspects of the proteins specific to the disease condition can be idetified. The identity of each candidate proteins can be determined by protein microsequencing and database searches. Similar genetic linkage tests will be performed to each candidate gene by intragenic markers.

An alternative approach will be a whole canine genome scan to identify positional candidate genes. 341 highly polymorphic markers of all types have been placed on the 3rd generation dog genome maps with average resolution of 9.0 cM and 95% coverage of the genome. Recently, a high resolution radiation hybrid map with 400 markers has been produced and partially integrated with the linkage map. Informative type I markers will be tested for linkage to canine I-GS. Candidate genes will be identified by comparing the linked region to respective human and mouse genome maps and can be further screened by examining the function and tissue expression of each gene. Candidates will be tested for linkage to the canine disease locus with intragenic markers.

The linked gene identified by either method mentioned above will be cloned and examined for the disease-causing mutations by comparing the gene sequence between the normal and affected dogs. The defined mutation(s) will be further confirmed by checking for the mutation(s) in the normal dog population and functional studies on the gene from both normal and affected dogs.