BRIEF REPORT

Genetic Evidence of an Accessory Activity Required Specifically for Cubilin Brush-Border Expression and Intrinsic Factor-Cobalamin Absorption

By Danbin Xu, Renata Kozyraki, Thomas C. Newman, and John C. Fyfe

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 CUBN cDNA was cloned and sequenced, showing high identity with human and rat CUBN cDNAs. An intragenic CUBN marker was identified in the canine family and used to test the hypothesis of genetic linkage of the disease and CUBN loci. Linkage was rejected, indicating that the canine disorder resembling Imerslund-Gräsbeck syndrome is caused by defect of a gene product other than cubilin. 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.

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Gastrointestinal cobalamin (vitamin B₁₂) absorption is a complex and highly specific process for assimilation of a dietary nutrient essential for normal hematopoiesis and integrity of the central nervous system. Imerslund-Gräsbeck syndrome (I-GS) is a rare autosomal recessive disorder, originally described in Norway1 and Finland,2 that is characterized by selective cobalamin malabsorption, leading to juvenile-onset severe megaloblastic anemia, and proteinuria. The disorder was mapped to a locus on chromosome 10p12.1 (MGA 1) in Finnish, Norwegian, and Saudi Arabian kindreds.3,4 The intrinsic factor-cobalamin (IF-cbl) receptor is a 460-kD apical brush-border multiligand-binding protein functioning in distal small intestinal and renal proximal tubule epithelia5-10 and was an obvious candidate gene for I-GS. The IF-cbl receptor was recently named cubilin in recognition of its unique domain structure,7 and the human gene locus (CUBN) was mapped within the MGA 1 locus.11 Accordingly, 2 disease-specific mutations in the CUBN locus were recently demonstrated in Finnish kindreds.4

A canine model of autosomal recessive I-GS has been described in which immunoelectron microscopy and cell fractionation studies demonstrated failure of cubilin expression in apical brush border membranes of ileum and renal cortex, whereas other brush-border proteins were expressed normally.12,13 Similar to I-GS patients, affected dogs develop hematologic and metabolic signs of selective cobalamin deficienc

MATERIALS AND METHODS

The parents (members of a breeding colony maintained at Michigan State University, East Lansing, MI) and 23 offspring, including 13 affected and 10 clinically normal dogs, of matings between an affected female and an obligate carrier male were studied. The disease phenotype of each was determined by monitoring puppies until 12 to 16 weeks of age without parental cobalamin administration for growth and laboratory abnormalities previously described18 and the same parameters for 3 to 4 weeks after parental cobalamin administration.

Peptide sequence was obtained from canine renal cubilin purified, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane as described.13 In situ protease digestion and peptide sequencing were performed by the Protein Chemistry Facility of the Worcester Foundation for Experimental Biology (Shrewsbury, MA). A canine renal tubule cDNA library16 was screened with a rat and canine partial cDNA probes by standard methods.17 Hybridizing clones containing overlapping inserts were sequenced on both strands by automated dideoxy termination cycle sequencing methods (ABI 373A Sequencer; Applied Biosystems, Inc, Foster, CA). Position 1 of the canine cubilin cDNA refers to the first nucleotide of the full-length cDNA; the A residue of the first ATG is nucleotide 74. (Canine CUBN nucleotide sequences reported here have been submitted to GenBank: full-length cDNA, accession no. AF137068; partial genomic DNA, accession no. AF137069.)
To locate a segregating canine CUBN variation for genotyping, the CUBN intron/exon structure was partially determined by polymerase chain reaction (PCR) and sequencing. Genomic DNA was isolated from liver or blood samples by standard methods,17 and portions of the CUBN gene were amplified using various combinations of the primers 838F, 5'-AGGCTGCTGGGTCAATGAGC-3', 947F, 5'-GGCTGGCAGAAGGAATTTGATAGT-3'; 1150R, 5'-TGCTGGCGCCAGCTCGGATTAGG-3'; and 1341R, 5'-CAGCCCCAACCTGATCTACG-3'. PCR reactions of 50 µL contained 1× PCR buffer, 0.4 mmol/L of each deoxynucleotide, 0.5 µmol/L of each primer, and 500 ng of genomic DNA template. TaKaRa LA Taq polymerase (2.5 U; PanVera Corp, Madison, WI) was added after 2 minutes at 94°C, and reactions were performed for 35 cycles of 98°C for 20 seconds and 68°C for 20 minutes. Oligonucleotide primer sets were designed to amplify CUBN allele-specific products using primers flanking the 17-bp insertion and that the clinically normal sire, heterozygous at the I-GS disease locus, was determined by the following criteria: affected dogs failed to gain weight after 9 to 13 weeks of age; they had low serum cobalamin concentrations, mild megaloblastic dysmaturity, methylmalonic aciduria, and proteinuria; and all abnormalities other than proteinuria were reversed by parenteral cobalamin administration. In this family, canine I-GS is a fully penetrant, autosomal recessive trait.12 Therefore, affected offspring from these matings are homozygous and clinically normal littermates are heterozygous at the I-GS disease locus. CUBN genotyping was performed by PCR amplification of allele-specific products using primers flanking the 17-bp CUBN variation. PCR confirmed that the affected dam, homozygous at the disease locus, was homozygous for the intronic CUBN insertion and that the clinically normal sire, heterozygous at the disease locus, was heterozygous at the CUBN locus (Fig 1B). 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 ($\chi^2 = .39$, df = 1, P = .53). A disease trait locus and a
marker locus on a different chromosome, or located far from the disease locus on the same chromosome, recombine randomly (at a frequency of 0.5 in large samples) and segregate independently during meiosis. Thus, in contrast to I-GS reported in some human kindreds, the linkage data above indicate that canine I-GS in this kindred is not caused by mutation of \textit{CUBN} or any gene within 50 recombination units either side of the canine \textit{CUBN} locus. (A recombination unit [equal to 1% recombination and called a centiMorgan] in mammals is equivalent, on average, to \~{}1 Mb of DNA.)

But still, affected dogs lack apical brush-border expression of cubilin in ileal and renal epithelia,\textsuperscript{13} causing selective cobalamin malabsorption\textsuperscript{14,15} and selective proteinuria,\textsuperscript{8} respectively. Therefore, the absence of genetic linkage of the canine \textit{CUBN} marker to canine I-GS indicates there must be some other gene, distant from \textit{CUBN}, the product of which is required for normal cubilin folding, exit from the ER, and/or transport to the brush-border. 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 \textit{CUBN} locus. Human I-GS remains to be mapped in kindreds of various ethnic backgrounds.

There are at least 2 genes whose products associate with cubilin\textsuperscript{6,7} that are not linked to the human \textit{MGA 1} locus and defects of which could explain our findings and/or be implicated in some forms of human I-GS. The endocytic receptor, megalin (gp330), is postulated to anchor cubilin in the apical membrane and to facilitate endocytosis of cubilin-ligand complexes.\textsuperscript{7} Receptor-associated protein (RAP) is a molecular escort of the lipoprotein receptor-related proteins, including megalin, and may also facilitate proper folding of cubilin within the ER.\textsuperscript{18} Alternatively, there may be activities of other gene products, yet to be determined, that are specifically required for ER-export competence and brush-border expression of cubilin.

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