Cubilin and megalin expression and their interaction in the rat intestine: effect of thyroidectomy

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Yammani, Raghunatha R., Shakuntla Seetharam, and Bellur Seetharam. Cubilin and megalin expression and their interaction in the rat intestine: effect of thyroidectomy. Am J Physiol Endocrinol Metab 281: E900–E907, 2001.—Cubilin is a 460-kDa multipurpose, multidomain receptor that contains an NH2-terminal 110-residue segment followed by 8 epidermal growth factor (EGF)-like repeats and a contiguous stretch (representing nearly 88% of its mass) of 27 CUB (initially found in complement components C1r/C1s, Uegf, and bone morphogenetic protein-1) domains. Cubilin binds to intrinsic factor (IF)-cobalamin (cbl, vitamin B12) complex and promotes the ileal transport of cbl. The 460-kDa form of cubilin is the predominant form present in the apical brush-border membranes of rat intestine, kidney, and yolk sac, but a 230-kDa form of cubilin is also noted in the intestinal membranes. In thyroidectomized (TDX) rats, levels of intestinal brush-border IF-[^57]Co]-labeled cbl binding, 460-kDa cubilin protein levels and tissue (kidney) accumulation of cbl were reduced by ~70%. Immunoblot analysis using cubilin antiserum of intestinal total membranes from TDX rats revealed cubilin fragments with molecular masses of 200 and 300 kDa. Both of these bands, along with the 230-kDa band detected in the total membranes of control rats and unlike the 460-kDa form, failed to react with antiserum to EGF. Mucosal membrane cubilin associated with megalin was reduced from ~12% in control to ~4% in TDX rats, and this decreased association was not due to altered megalin levels. Thyroxine treatment of TDX rats resulted in reversal of all of these effects, including an increase to nearly 24% of cubilin associated with megalin. In vitro, megalin binding to cubilin occurred with the NH2-terminal region that contained the EGF-like repeats and CUB domains 1 and 2 but not with a downstream region that contained CUB domains 2–10. These studies indicate that thyroxine deficiency in rats results in decreased uptake and tissue accumulation of cbl caused mainly by destabilization and deficit of cubilin in the intestinal brush border.

Vitamin B12; transport

GASTROINTESTINAL UPTAKE AND TRANSPORT of dietary cobalamin (cbl, vitamin B12) occur bound to gastric intrinsic factor (IF) by receptor-mediated endocytosis via an apically expressed receptor for IF-cbl (24). The receptor originally known as IF-cbl receptor is now renamed cubilin due to the presence of 27 CUB (initially found in complement components C1r/C1s, Uegf, and bone morphogenetic protein-1) domains that are contiguous and represent nearly 88% of its total mass. Upstream of the 27 CUB domains, cubilin contains a 110-residue variable region, followed by eight EGF-like repeats. This multidomain receptor (19) with a molecular mass of 460 kDa binds to a variety of proteins including high-density lipoprotein (HDL) (11), albumin (2), megalin, and receptor-associated protein (19). Recently, it has been demonstrated (10) that cubilin interaction with megalin, a 600-kDa endocytic receptor, is essential for the endocytosis of HDL in rat yolk sac carcinoma cells. Moreover, megalin expression in these cells is suggested to be critical for cubilin expression at the cell surface (10). Thus a close association of cubilin with megalin in cultured cells may be important for both endocytosis and their trafficking from the endoplasmic reticulum. Despite these studies, it is not known whether a cubilin-megalin association exists in the intact intestine, the site of dietary cbl absorption, and whether such an association is important for cbl absorption and transport.

Previous studies (3) have shown that binding of IF-cbl to the intestinal mucosa and cbl transport are decreased in TDX rats, and a number of human studies (1, 7, 9) have demonstrated development of anemia in a majority of hypothyroid patients. Although the cause of anemia in these patients is not known, it is likely to be due to the development of a cbl deficiency caused by malabsorption of cbl. This hypothesis was tested in the present studies with the use of thyroidectomized (TDX) rats as an experimental model. The results of our study show that thyroxine deficiency in rats leads to impaired uptake and transport of cbl due to poor expression of cubilin at the cell surface. In addition, our study also demonstrates that, in TDX rats, intestinal cubilin undergoes degradation, losing its NH2-terminal region, including the EGF-like repeats, which results in its decreased ability to bind megalin.

MATERIALS AND METHODS

Materials. The following were commercially purchased from the sources indicated: [^57]Co-cbl (1.3 μCi/μg) and carrier-

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renal accumulation of [57Co]cbl are based on our earlier segments. The segments were exposed to 5 mM KPO4 buffer, removed from the pylorus to the ileocecal end and cut into label, the animals were killed. The intestine was carefully rats through a feeding tube. One hour after instillation of the in phosphate-buffered saline. The mucosa was scraped and surface-bound radioactivity. The segments were then washed pH 5.0, containing 5 mM EDTA for 10 min to remove the intestinal mucosa, yolk sac, or kidney were prepared by the total membranes. Apical brush-border membranes from in-ferred overnight at 4°C onto Immobilon-P membrane by use of a constant voltage of 30 V. The membranes were then probed with diluted (1,5,000) antiserum to rat cubilin, megalin, or human EGF. The immunoblots were quantified using the Ambis-radioimaging system, and the intensity of the immunoreacting bands was translated into arbitrary units. The linearity of the band intensity was confirmed with immu-noblots generated using pure rat renal cubilin (200–2,000 ng). The immunoblots (Figs. 1, 2, and 5) are representative data from three separate blotting experiments with mem-branes isolated from four to five animals in each group.

Megalin-cubilin interactions. Isolated total membranes (100 μg protein) from control, TDX, and thyroxine-treated TDX rats were incubated with IF-[57Co]cbl (5 pmol) for 2 h at 22°C in 1 ml containing 5 mM Tris-HCl buffer containing (in mM) 140 NaCl 0.1 PMSF, and 2 benzamidine. The membrane-bound radioac-tivity obtained by centrifugation at 20,000 g was solubilized in 1 ml of buffer A containing Triton X-100 (1%). The Triton-solubilized fraction was incubated for 2 h at room temperature with either preimmune serum or antiserum (6 μl) to rat IF, cubilin, or megalin. The immune complex was precipitated with protein A-Sepharose, and the [57Co]cbl bound to protein A was measured using a Beckman γ-counter.

Binding of NH2-terminal fragment of cubilin with megalin. The NH2-terminal fragment (which contains 110-amino acid residue, NH2-terminal, eight EGF-like domains, and CUB domains 1 and 2) and a downstream fragment containing CUB domains 2–10 were obtained by reverse-transcribed polymerase chain reaction. The NH2-terminal fragment of cubilin was amplified (2 kb) using the forward primer 5'-ATGTCCTCGAGCTTT-3', corresponding to nucleotides 1–15, and the reverse primer 5'-AATGACTCGACCAAG-3', corresponding to nucleotides 1922–1936. The CUB 2–10 region of the cubilin was amplified (2.6 kb) with forward 5'-GGGCGGATCTGA-3' and reverse 5'-ACTTTCAACT-TCAA-3' primers, corresponding to nucleotides 1769–1783 and 4997–5011, respectively. Amplified products were purifi-cated by means of a gel extraction kit (Millipore). The PCR products were subcloned into the expression vector (pSec B; Invitrogen, Carlsbad, CA). The authenticity of the two cubulin fragments amplified was confirmed by sequencing and by their ability to bind the ligand IF-cbl and to react with polycubilin antiserum (unpublished observations). The plasmid was translated in vitro with the TNT quick-coupled transcription/translation system from Promega (Madison, WI). The 35S-translated product was used for binding to the affinity matrix containing bound rat megalin.

Other methods. Total RNA from different groups was iso-lated according to Chomczynski and Sacchi (6). Total RNA (25–50 μg) was used for blotting, and the blot was probed with a 32P-labeled, 2.1-kb cubilin fragment (NH2 terminus) generated by PCR. Protein estimation in membrane samples was carried out according to Bradford (4). Cubilin activity in the intestinal brush borders was measured by its ability to bind the IF-[57Co]cbl complex, as described earlier (28). Briefly, rat IF-[57Co]cbl (40–2,000 pg) was incubated with 25–50 μg of intestinal brush-border membrane protein in the presence of 10 mM Tris·HCl buffer, pH 7.4, containing 5 mM of either CaCl2 or Na2EDTA. The Ca2+ specific binding of the ligand was calculated as before (26).

RESULTS

Cubilin protein expression and uptake of IF-[57Co]cbl. Immunoblot analysis of apical brush-border membrane isolated from intestinal mucosa, kidney, and yolk sac revealed a predominant protein band with a molecular mass of 460 KDa. However, in the intestine...
apical membranes, another protein band, with a molecular mass of 230 kDa, was identified; this band was very faint in the intestinal total membranes but was absent in the apical membranes of kidney and yolk sac (Fig. 1). In addition to the 460-kDa band, a very high molecular mass band that reacted with cubilin antisem was also detected in the kidney and yolk sac membrane, and the intensity of this band was weak in the intestine. A faint band with a lower mass of ~200 kDa, which was not observed in the yolk sac membranes, could also be seen in the intestinal and renal apical brush-border membranes.

When immunoblot analysis of the intestinal apical brush-border membranes isolated from control and TDX rats was carried out (Fig. 2, top), the levels of the 460-kDa form of cubilin were drastically reduced, and in some rats it was hardly identifiable. However, the intensity of the 460-kDa cubilin band increased after treatment of TDX rats with thyroxine. To determine whether the loss of cubilin expression in the apical brush border in TDX animals was due to loss of total mucosal cubilin, immunoblotting was carried out using higher amounts (50 μg) of total membrane protein (Fig. 2, bottom). Both the 460- and the 230-kDa forms of cubilin were identified in control rats (lane 1), and in the total membranes of TDX animals, both of these protein bands disappeared, and the molecular masses of immunocross-reactive bands were 200 and 300 kDa (lane 2). Upon treatment with thyroxine, the 460-kDa band reappeared and was the predominant band (lane 3). These results suggest that, in thyroxine deficiency, levels of the 460- and 230-kDa forms of cubilin are altered due to their degradation. To confirm this and to examine the potential degradation of cubilin from the NH₂-terminal end, the total membranes were subjected to immunoblot analysis with the use of EGF antiserum (Fig. 2, bottom). Although the total membranes from control rats reacted with EGF antiserum, demonstrating a single band of 460 kDa (lane 4), this band was absent in membranes obtained from TDX rats (lane 5).

When the immunoreactive cubilin bands observed in either intestinal brush border or total membranes were
quantified (Fig. 3), it was clear that TDX actually had no significant effect on the total membrane cubilin protein levels (Fig. 3, bottom), but it decreased cubilin protein levels in the apical brush border (Fig. 3, top). Furthermore, TDX had no effect on either the IF-cbl binding activity of cubilin or cubilin protein levels in the renal brush-border membranes of the rat (data not shown). Taken together, these observations suggest that TDX affected cubilin protein levels in the intestinal brush-border membrane and, as such, had no effect on the total mucosal cubilin protein levels. This observation is supported by dot blot hybridization, which showed that altered thyroid status in rats had no significant effect on cubilin mRNA levels (Fig. 4). Due to an extremely low abundance of cubilin transcript in the intestine in general and in rat intestine in particular, more sensitive quantitative Northern blotting of cubilin mRNA levels was not possible.

To further examine whether cubilin protein deficit in the intestinal apical brush borders had indeed any effect on the IF-[57Co]cbl binding activity, uptake, and plasma transport of cbl, the ligand IF-[57Co]cbl was administered orally, and the accumulation of [57Co]cbl in the kidney was studied. The results (Table 1) show that, in vitro, IF-[57Co]cbl binding activity in the brush border decreased by ~70% from 66 to 20 fmol/mg protein. This decline in IF-[57Co]cbl binding activity also resulted in a similar decline in the in vivo uptake of IF-cbl from 60 to 18 fmol/mg protein and in cbl accumulation in the kidney from 2.8 to 0.7 fmol/mg protein. These results clearly indicate that thyroxine deficiency results in decreased intestinal mucosal uptake and tissue accumulation (kidney) of orally administered cbl.

Megalin expression in the intestine and its interaction with cubilin. Earlier in vitro studies (19) had shown that purified cubilin bound to megalin, and in cultured yolk sac cells, megalin association with cubilin was suggested to be important for the endocytosis of HDL and that synthesis of megalin was required for the cell surface expression of cubilin (10). Thus we wanted to examine first whether the cubilin-megalin association occurred in the intact intestinal tissue and if so, whether this association was affected in TDX animals. Our initial immunoblot studies using the apical brush-border membrane (Fig. 5A) from the proximal and distal regions of the adult rat intestine revealed that megalin expression in rat intestine was limited to the distal regions. Furthermore, megalin levels in the distal intestinal apical brush-border membranes (Fig. 5B) or in the total membranes (Fig. 5C) did not change significantly in control (lanes 1 and 4), TDX (lanes 2 and 5), or thyroxine-treated TDX rats (lanes 3 and 6). Similar to intestine, renal megalin levels also did not change significantly in any of the three groups of rats (data not shown).

Because cubilin association with megalin was suggested to be essential for the endocytosis of ligands bound to cubilin, we initially examined whether such an association exists in the intestinal mucosa membranes. When the IF-[57Co]cbl radioactivity bound to total membranes of the normal rat was extracted and treated with antisera to megalin, nearly 12% of the radioactivity in rat intestine was limited to the distal regions. Furthermore, megalin levels in the distal intestinal apical brush-border membranes (Fig. 5B) or in the total membranes (Fig. 5C) did not change significantly in control (lanes 1 and 4), TDX (lanes 2 and 5), or thyroxine-treated TDX rats (lanes 3 and 6). Similar to intestine, renal megalin levels also did not change significantly in any of the three groups of rats (data not shown).

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antiserum to IF or cubilin was used for immunoprecipitation experiments, nearly 80–85% of the radioactivity extracted from the apical brush-border membrane was precipitated.

The observation that, in TDX rats, cubilin did not react with antiserum to EGF (Fig. 2, bottom) and that its association with megalin was impaired suggested that the NH$_2$-terminal region of cubilin, including the EGF repeats and CUB domains 1 and 2, may be involved in the interaction with megalin. To test this directly, cubilin cDNA fragments containing the 110-residue amino-terminal end that also included the eight EGF-like repeats and CUB domains 1 and 2 and a downstream fragment containing CUB domains 2–10 were translated in vitro. SDS-PAGE analysis of the labeled translated products (Fig. 7) revealed that, in both cubilin fragments, the CUB 2–10 domain (lane 1) and the translated NH$_2$-terminal (lane 2) and the molecular mass of the synthesized proteins, the 100- and 71-kDa values were very close to the expected values of 110 and 74 kDa, respectively. SDS-PAGE analysis (Fig. 7) of the radioactivity bound to megalin revealed that the amino-terminal fragment of cubilin bound to megalin in the presence of Ca$^{2+}$ (lane 3) but not in the presence of EDTA (lane 4). The CUB 2–10 fragment did not bind to megalin in the presence of either Ca$^{2+}$ (lane 5) or EDTA (lane 6).

**DISCUSSION**

In this study, the presence of different molecular forms of cubilin in the rat intestinal apical brush border and total membranes was first determined to assess the effect of TDX on their levels and potential association with megalin. Earlier studies (reviewed in Ref. 25) had identified the molecular mass of intestinal cubilin in various species to be ~200 kDa. However, a recent study in canine intestine (34) and the present studies in rat intestine have identified the 460-kDa form of cubilin to be the predominant form expressed in intestinal (Fig. 1) as well as in renal and yolk sac brush-border membranes. In addition to the 460-kDa form, a very high molecular mass band (Mr > 10$^6$) was also noted in all of the rat tissue apical membranes. These observations are in agreement with 1) the size of cubilin predicted on the basis of its cDNA sequence (19) and 2) the demonstration that, in vivo, cubilin is assembled as a noncovalent trimer connected by an NH$_2$-terminal coiled-coil helix (16). Regarding the presence of a 230-kDa cubilin form in the rat intestinal brush borders and its absence in kidney and yolk sac membranes, several lines of evidence suggest that it is formed in the intestinal brush border by the in situ action of extracellular pancreatic proteases that are present in this tissue but are absent in kidney and yolk sac. First, cubilin with a molecular mass of ~200 kDa has also been observed in canine intestinal brush borders (34). Second, in vitro studies have shown that trypsin degrades bovine cubilin into several fragments (16). Third, in metabolically labeled, polarized epithelial intestinal Caco-2 (22) and renal opossum kidney (21) cells that lack proteases, a single cubilin band was noted in the apical brush-border membrane. Fourth, cubilin purified from canine intestine in the absence of proteolytic enzyme inhibitors generated several func-

**Table 1. Effect of thyroidectomy on intestinal brush border on IF-[57Co]Cbl binding and in vivo mucosal uptake and renal accumulation of [57Co]Cbl**

<table>
<thead>
<tr>
<th>Animals and Treatment</th>
<th>IF-[57Co]Cbl Binding</th>
<th>Mucosal Uptake, fmol/mg protein</th>
<th>Kidney Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated control</td>
<td>66 ± 5</td>
<td>60 ± 6</td>
<td>2.8 ± 0.20</td>
</tr>
<tr>
<td>Thyroidectomized</td>
<td>20 ± 1</td>
<td>18 ± 1.5</td>
<td>0.70 ± 0.8</td>
</tr>
<tr>
<td>Thyroidectomized + treated with thyroxine</td>
<td>63 ± 6</td>
<td>59 ± 6.1</td>
<td>2.4 ± 0.15</td>
</tr>
</tbody>
</table>

Values are means ± SD from 6–8 rats. Cubilin activity was determined using apical brush-border membrane and rat gastric intrinsic factor (IF)-[57Co]cobalamin (Cbl). Mucosal uptake of ligand IF-[57Co]Cbl was determined 1 h after oral administration of rat IF-[57Co]Cbl (3.5 pmol). The amount of [57Co]Cbl accumulated in the kidney was determined 6 h after oral administration of the ligand.
soon after its delivery to this site. Additional studies indicate that intestinal cubilin becomes unstable in hypothyroidism (Fig. 2, bottom lane 2) of TDX rats and that its degradation to form the smaller-sized fragments occurred from the NH2-terminal end that includes the EGF-like repeats. Thus it is likely that the 230-kDa form of cubilin may be a major intermediate during the degradative pathway of the 460-kDa form and that the degradative process may be initiated in the brush borders in situ involving pancreatic proteases. Additional studies will be required to determine the exact location of the site of intestinal cubilin that generates the 230-kDa form in normal rat (Fig. 1) or its further degradation in thyroxine deficiency (Fig. 2, bottom).

The effects of hypothyroidism on IF-[57Co]cbl binding to brush border is specific to the intestinal tissue, as neither the binding activity nor the cubilin protein levels were affected in the rat kidney brush borders.
(data not shown). It is interesting to note that similar intestinal-specific changes have also been demonstrated during hypothyroidism for other proteins, such as carbonic anhydrase and Mg$^{2+}$/HCO$_3$ ATPase (32). In addition to intestinal-specific effects on some proteins, TDX also causes other tissue-specific changes as noted with insulin-like growth factor receptor, whose activity in the anterior pituitary, but not in brain, liver, and renal cortex (18), is affected. Thus tissue specificity of changes in specific protein levels or their membrane function in altered thyroid status may depend on whether the effects are direct on the protein itself or whether they are indirect, caused by TDX-induced changes in membrane fluidity. Thus the possibility exists that the decreased plasma membrane transport of cbl noted in this study could be due to TDX-induced changes in membrane fluidity. This consideration is relevant, because it is well recognized that altered thyroid status, particularly hypothyroidism in rats, is known to increase the fluidity of both intestinal (5) and renal (20) plasma membranes. However, this possibility is highly unlikely for the following reasons. First, there is no evidence that altered fluidity in the brush border increases the susceptibility of cubilin to endogenous proteases. Second, on the basis of its cDNA-predicted structure, cubilin has no transmembrane domain (19); thus it is highly unlikely that lipid order changes could affect its stability and activity. Third, the trans-cbl II receptor, which facilitates the uptake of absorbed cbl from the circulation bound to plasma trans-cbl II, is not altered in TDX rats (3). Finally, TDX-induced posttranscriptional destabilization and modification are not unique to cubilin, as rat liver glucocorticoid receptor has also been demonstrated (15) to undergo similar changes in TDX rats.

The functional consequence of TDX due to cubilin deficit in the apical brush border resulted in decreased mucosal uptake of the ligand IF-[^57Co]cbl and in subsequent accumulation of[^57Co]cbl in the kidney (Table 1). If decreased uptake of IF-cbl and tissue (kidney) levels of cbl can be demonstrated with a single oral dose of cbl, it is likely that, in the absence of thyroxine treatment, these animals may eventually develop cbl deficiency. This suggestion is substantiated by a number of clinical studies that have noted the development of anemia in adult (8), child, and adolescent patients with hypothyroidism. Although these studies did not address the cause of anemia in these patients, there is some evidence from patients with autoimmune hypothyroidism (9) that absorption of cbl, as determined by the Schilling test, was impaired. It is likely that the development of cbl deficiency in these patients is slow to develop and that the extent of the deficiency may depend on the degree of hypothyroidism. Our studies (Fig. 2, top) indicate that cubilin deficit or its recovery in the brush border upon treatment with thyroxine may vary among rats.

Our immunoblot and immunoprecipitation studies show that, like cubilin (30), megalin is also expressed in the distal intestine (Fig. 5A), a site of active endocytosis (33), and that cubilin and megalin are associated in native tissue membranes. Our data indicating an association of only 12% of cubilin with megalin in control rat intestinal mucosa may not be completely quantitative, because we were measuring only the immunoprecipitation of the ligand IF[^57Co]cbl bound to the cubilin-megalin complex. It is not known whether all of the antigenic sites of megalin were exposed and thus were accessible for antibody recognition, particularly when it exists as a complex with cubilin bound to the ligand. Despite this uncertainty, when similar immunoprecipitation studies were carried out with the total membranes from TDX rats and thyroxine-treated TDX rats (Fig. 6), the cubilin associated with megalin was 4 and 24%, respectively. This clearly indicates that the modulation of cubilin levels was responsible for the noted difference in the percentage of its association with megalin, since megalin levels in either the intestinal apical brush border or total membranes were not altered. However, it is not clear at the present time why the percentage of cubilin associated with megalin in thyroxine-treated rats rose to 24%, which is nearly twice the value of 12% noted in control animals. Because there was no significant effect of TDX or thyroxine treatment of TDX rats on megalin levels, it is likely that thyroxine treatment may affect steady-state cubilin levels by increasing its intracellular stability so that more cubilin would be bound to megalin.

Although our immunoprecipitation data clearly indicate the formation of a megalin-cubilin-IF-cbl complex in the native apical intestinal membranes, confirmation of these findings at the ultrastructural level has been hampered due to technical difficulties. These include extremely low levels (fmol) of cubilin and IF bound to it (12) and the difficulty of interpretation of the data due to the intense mucosal background staining obtained with the megalin antibody (17). Despite this limitation, the association of cubilin and megalin has been demonstrated by ultrastructural studies in the apical invagination and microvilli of rat renal proximal tubule, yolk sac (19), and endocytic vesicles of yolk sac endoderm-like cells (11), where the levels of these two proteins are much higher relative to the intestinal tissue.

The observation that the NH$_2$-terminal region, including the eight EGF-like repeats and CUB domains 1 and 2, is involved in megalin binding (Fig. 7) strongly suggests that disruption of the cubilin-megalin interaction is due to loss of this region in intestinal cubilin of TDX rats. To the best of our knowledge, this is the first report that identifies the existence of a cubilin-megalin complex in native intestinal membranes as well as the region of cubilin that interacts with megalin. Additional studies are needed to further dissect the role of individual regions of this fragment (i.e., NH$_2$-terminal 110 residue, the eight EGF repeats, or CUB domains 1 and 2) that may be important in megalin binding. The loss of this region due to destabilization of cubilin in TDX rats could result in a loss of megalin binding and could occur both on the cell surface after its delivery and within the cells. If it occurred intracellularly, then failure to bind megalin might result in
poor trafficking of cubilin from the endoplasmic reticulum. Further studies are needed to address these issues.

In conclusion, the results of our study show that, in TDX rats, 1) absorption and tissue accumulation of orally administered cbl is inhibited due to a deficit of cubilin in the apical brush borders; 2) cubilin is destabilized, causing its progressive degradation; and 3) the cubilin-megalin association is impaired due to loss of the NH2-terminal region (including the EGF repeats), a region involved in megalin binding. Further studies are required to address the site and cause of increased sensitivity of intestinal cubilin in TDX rats.

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