The soluble intracellular domain of megalin does not affect renal proximal tubular function *in vivo*

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Megalin-mediated endocytic uptake constitutes the main pathway for clearance of plasma proteins from the glomerular filtrate in proximal tubules. Little is known, however, about mechanisms that control megalin expression and activity in the kidney. A widely discussed hypothesis states that upon ligand binding a regulated intramembrane proteolysis releases the cytosolic domain of megalin and this fragment subsequently modulates *megalin* gene transcription. Here, we tested this by generating a mouse model that co-expressed both the soluble intracellular domain and full-length megalin. Despite pronounced synthesis in the proximal tubules, the soluble intracellular domain failed to exert distinct effects on renal proximal tubular function, including *megalin* expression, endocytic retrieval of proteins, or global renal gene transcription. Hence, our study argues that the soluble intracellular domain does not have a role in regulating the activity of megalin in the kidney.

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Megalin is the most important endocytic receptor responsible for bulk clearance of low-molecular-weight proteins from the glomerular filtrate in the renal proximal tubules.[1] In particular, receptor-mediated uptake of filtered plasma carriers such as vitamin D-binding protein[2] or retinol-binding protein[3] prevents uncontrolled loss of lipophilic vitamins and regulates systemic vitamin homeostasis. The importance of megalin for renal resorptive capacity is underscored by low-molecular-weight proteinuria observed in megalin-deficient mice[4] and in patients with inheritable megalin gene defects.[5] Malfunction of the receptor has also been implicated in diseases affecting the proximal tubule, including Dent's disease,[6,7] renal Fanconi syndrome,[4] and aminoglycoside-induced nephrotoxicity.[8] Besides acting as clearance pathway for filtered metabolites, megalin also regulates functional expression of other transporters in the proximal tubules, including cubilin,[9,10] type IIa sodium phosphate cotransporter (NaPi-IIa),[11] and Na^+/H^+ exchanger isoform 3 (NHE3).[12]

Given the central role of megalin for tubular resorptive processes, remarkably little is known about mechanisms that regulate renal receptor expression. Recently, an interesting hypothesis has been advanced that regulated intramembranous proteolysis liberates the intracellular domain (ICD) of megalin that, in turn, may act as negative regulator of receptor gene transcription.[13–15] Although intriguing, this model is based on experimental evidence obtained in cultured cells and still requires rigorous testing *in vivo*. Now, we have generated a novel mouse model expressing the megalin ICD under control of the endogenous *megalin* promoter, and we have explored the relevance of the soluble receptor domain as regulator of receptor function.

RESULTS

We have generated a mouse model that expresses the soluble ICD of megalin instead of the full-length form of the receptor from the endogenous *megalin* locus (Figure 1a and b, Supplementary Figure S1). Robust expression of the ICD was observed in kidneys from adult mice carrying one copy of the transgene ($megalin^{+/TgICD}$) using western blotting

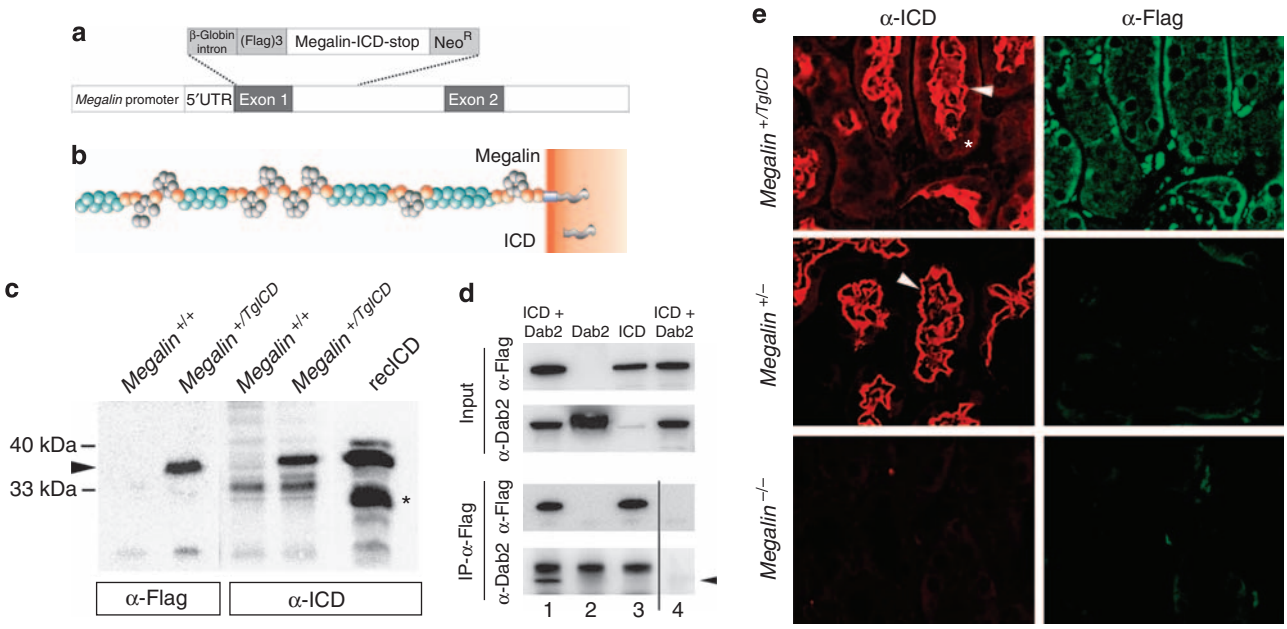

Figure 1 | Generation of mice expressing the intracellular domain of megalin. (**a**) Design of the targeting vector replacing exon 1 and 3.6 kb of intron 1 of the mouse *megalin* locus with a mini gene construct encoding an intron of the rabbit β-globin gene followed by three copies of the flag epitope, the intracellular domain (ICD) of megalin, and a translation stop codon. Neo^R was used as a marker to select for murine embryonic stem cells carrying the mini gene. Targeting of the wild-type *megalin* locus results in expression of the flag-tagged soluble ICD under control of the endogenous *megalin* promoter. (**b**) Structure of full-length megalin and the soluble ICD. The ICD encompasses amino acids 4448 to 4661 of the mouse megalin polypeptide (NM_001081088). (**c**) Western blot analysis detects expression of the ICD (arrowhead) in cytosolic kidney extracts from adult *megalin*$^{+/TgICD}$ but not in *megalin*$^{+/+}$ mice. Both antisera against the flag epitope (α-Flag) and the ICD (α-ICD) were used. The recombinant ICD transiently expressed in HEK293 cells was used as control (recICD). The asterisk marks a degradation product of recICD in HEK293 cell lysates. (**d**) CoIP (co-immunoprecipitation) of Disabled2 (Dab2) and the ICD from Chinese hamster ovary (CHO) cells. Panel input represents cell lysates from CHO cells expressing murine Dab2 (lane 2), the flag-tagged ICD (lane 3), or both proteins (lanes 1 and 4). Panel IP indicates detection of the ICD (α-Flag) and Dab2 (α-Dab2; arrowhead) in anti-Flag immunoprecipitates from the various CHO cell clones (lanes 1–3). CoIP of Dab2 with α-Flag is observed from cell with ICD (lane 1) but not from cells without ICD (lane 2). As negative control, non-immune immunoglobulin G (IgG) failed to precipitate ICD or Dab2 (lane 4). (**e**) Immunohistological detection of the cytoplasmic domain of megalin (α-ICD) or the flag-tagged ICD (α-Flag) in the kidneys of adult *megalin*$^{+/TgICD}$, *megalin*$^{+/-}$, or *megalin*$^{-/-}$ mice. The anti-ICD antiserum recognizes the carboxyl terminal domain of the full-length receptor at the apical cell surface of proximal tubular cells in *megalin*$^{+/-}$ and *megalin*$^{+/TgICD}$ mice (arrowheads). In addition, a diffuse cytoplasmic pattern for the soluble ICD is observed in the *megalin*$^{+/TgICD}$ kidneys (asterisk). Anti-Flag IgG detects only the recombinant ICD in renal *megalin*$^{+/TgICD}$ tissue. No specific staining for megalin or flag-ICD is observed in kidneys from *megalin*$^{-/-}$ animals (original magnification ×63).

(Figure 1c) or immunohistology (Figure 1e). In contrast to the wild-type receptor that localized to the apical surface of proximal tubular cells (PTCs), the ICD showed a diffuse cytoplasmic pattern in the very same cells (Figure 1e). Diffuse cytoplasmic localization was confirmed in kidneys from mouse embryos, homozygous for the knock-in construct (*megalin*$^{TgICD/TgICD}$ Supplementary Figure S2). Co-immunoprecipitation of the ICD with Disabled-2, an established adaptor for megalin with phosphotyrosine-binding domain,[16] confirmed the ability of the soluble receptor tail to bind physiological interaction partners (Figure 1d). Co-immunoprecipitation was also documented for the ICD with synectin, a PDZ-domain protein involved in trafficking of megalin in PTCs (data not shown).[17]

Mice with targeted *megalin* disruption (*megalin*$^{-/-}$) suffer from holoprosencephaly, a forebrain malformation that causes perinatal lethality of most affected individuals.[18,19] Only ~5% of homozygous mutants survive to adulthood.[2] The same phenotype was observed in mice homozygous for the *TgICD* allele (*megalin*$^{TgICD/TgICD}$) (Supplementary Figure S3). Therefore, we focused our analysis on the renal functions of the ICD on adult *megalin*$^{+/TgICD}$ animals.

Initially, we tested whether the ICD affects functional expression of *megalin* in the kidney. No discernable differences in expression level or in subcellular localization of megalin and its co-receptor cubilin in PTCs were observed by immunohistological analysis comparing *megalin*$^{+/TgICD}$ with *megalin*$^{+/-}$ kidneys (Figure 2a). No defect in uptake of ligands such as vitamin D-binding protein was evident (Figure 2a). In addition, *megalin*$^{+/TgICD}$ mice did not show low-molecular-weight proteinuria (Figure 2b). In contrast, excretion of ligands such as vitamin D-binding protein and retinol-binding protein was apparent in adult *megalin*$^{-/-}$ survivors (Figure 2b). Urinary electrolyte profiles (Na^+, K^+, Cl^-, Ca^{2+}, phosphate, glucose, and pH) were normal in *megalin*$^{+/TgICD}$ animals (not shown).

To quantify receptor activity more accurately, we established primary cultures from PTCs of the various genotypes

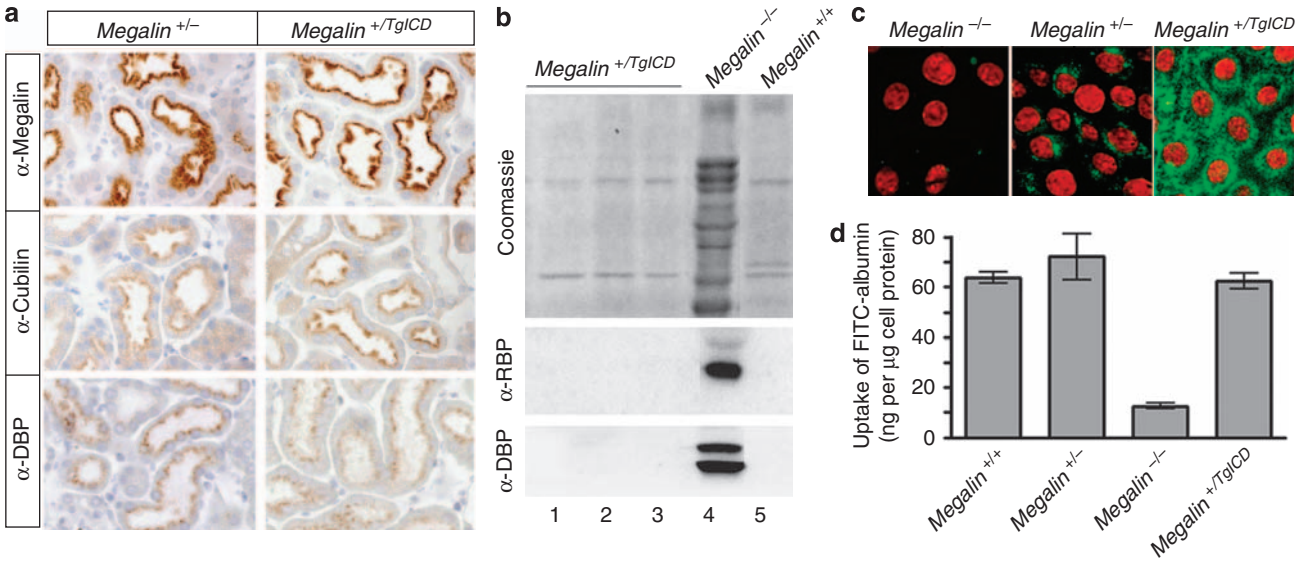

Figure 2 | The soluble intracellular domain does not interfere with endocytic activity of megalin in renal proximal tubules. (**a**) Immunohistological detection of full-length megalin, cubilin, and vitamin D-binding protein (DBP) on adult kidney sections from *megalin*$^{+/TgICD}$ and *megalin*$^{+/-}$ animals. No discernable differences are observed with respect to apical localization of megalin and its co-receptor cubilin, or presence of DBP in apical endosomes. (**b**) Western blot analysis of urine samples from adult mice of the indicated genotypes. Low-molecular-weight proteinuria (Coomassie) as well as excretion of DBP and retinol-binding protein (RBP) are observed in mice homozygous for the receptor null allele (*megalin*$^{-/-}$, lane 4) but not in control mice (*megalin*$^{+/-}$, lane 5) or animals heterozygous for *TgICD* (lanes 1–3). (**c**) Immunocytochemistry using anti-Flag immunoglobulin G (IgG) detects expression of the flag-tagged ICD (green) in primary proximal tubule cell cultures from *megalin*$^{+/TgICD}$ but neither from *megalin*$^{+/-}$ nor *megalin*$^{-/-}$ mice. Nuclei were counterstained with DraQ5. (**d**) Comparative analysis of endocytic uptake of fluorescein isothiocyanate (FITC)-labeled albumin in primary proximal tubule cell cultures from mice of the indicated genotypes. Similar rates of ligand uptake were observed in cells from *megalin*$^{+/+}$, *megalin*$^{+/-}$, and *megalin*$^{+/TgICD}$ animals, contrasting with the major defect (>80% inhibition) observed in *megalin*$^{-/-}$ cells. All values are mean ± s.e.m.

(Figure 2c). The activity of the megalin–cubilin pathway in *megalin*$^{+/TgICD}$ PTCs, as determined by albumin uptake,[10,20] was comparable to that of *megalin*$^{+/+}$ and *megalin*$^{+/-}$ cells (Figure 2d). This finding contrasted with the major endocytic defect observed in *megalin*$^{-/-}$ PTCs (Figure 2d).

Experimental evidence suggests a role for the ICD as negative regulator of *megalin* and *Nhe3* gene transcription in opossum kidney cells.[14] To test this hypothesis *in vivo*, we performed comparative transcriptome analysis in kidneys from *megalin*$^{+/TgICD}$ and *megalin*$^{+/+}$ animals. No significant alterations were observed by global gene expression profiling using mouse whole genome arrays (Figure 3a). The same finding was obtained comparing *megalin*$^{+/TgICD}$ and *megalin*$^{+/-}$ animals (not shown). In addition, no significant changes in the levels of *megalin* and *Nhe3* transcripts or megalin protein were detected using quantitative reverse transcriptase PCR and western blot analyses (Figure 3b).

DISCUSSION

Intramembranous proteolysis has been recognized as an important mechanism by which intracellular domains of cell surface receptors are released from the holoprotein to act as nuclear transcription factors. This concept has best been elucidated for Notch, a regulator of embryonic patterning.[21]

Intramembranous proteolysis has been reported for many transmembrane proteins, including megalin.[13] However, in contrast to Notch, the relevance of intramembranous proteolysis for most receptors remains enigmatic; one of the major obstacles being that it has been virtually impossible to detect ICDs *in vivo*. Thus, most studies rely on overexpression of recombinant ICDs in cell lines. As for megalin, ICD overexpression in opossum kidney cells resulted in a dramatic downregulation of *megalin* and *Nhe3* transcripts.[14] Accordingly, it has been discussed that release of the ICD, triggered by ligand binding, may reduce receptor activity and protect proximal tubules from harmful effects of protein overload.[14,15]

We have challenged the above hypothesis by generating a mouse model that expresses the ICD endogenously. The expression failed to rescue homozygous receptor mutants from developmental malformations (Supplementary Figure S3), a function proposed for the megalin ICD in nematodes.[22] In addition, the ICD did not affect renal megalin expression or function in adult mice co-expressing both proteins (Figure 2).

A dominant-negative effect of the ICD may have been anticipated based on the assumption that this domain may sequester adaptors (for example, Dab2 and synectin; Figure 1d) required for receptor trafficking.[16,17] In addition, yeast two-hybrid screens identified several transcription factors and signaling proteins interacting with the receptor tail.[23–25] Apparently, these interactions require binding to the full-length receptor in the context of the plasma membrane compartment to exert their physiological actions.

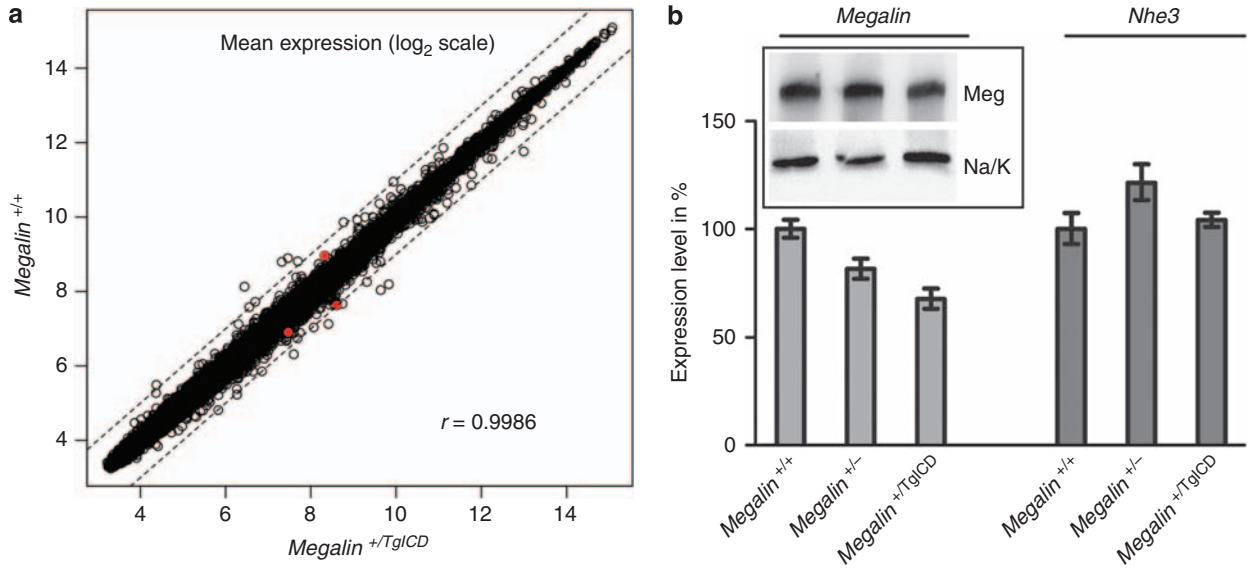

Figure 3 | The soluble intracellular domain of megalin does not affect the renal transcriptome. (**a**) Gene expression profiling of adult kidneys from *megalin*$^{+/+}$ and *megalin*$^{+/TgICD}$ animals using mouse whole genome arrays. Genome-wide mean expression levels (log_2 scale) are given for both genotypes. The dotted lines indicate twofold change in expression level and the red dots indicate significant changes (*P*-value <0.05, adjusted for multiple testing with the Benjamini and Hochberg correction). No statistically significant changes were observed for any gene at a threshold fold change of >2 and corrected *P*-value of <0.05. Rather, the expression profiles of the two samples were highly correlated (Pearson's correlation coefficient $r=0.9986$). (**b**) Quantitative reverse transcriptase PCR (RT-PCR) on total RNA isolated from adult kidneys of the indicated genotypes fails to detect significant changes in the level of transcripts encoding megalin and NHE3. Transcript levels were standardized to *β_2-microglobulin* transcripts as internal control. Data are given as percentage change in expression level compared with *megalin*$^{+/+}$ (set at 100%). Values are mean values ± s.e.m. The inset depicts western blot analysis of megalin (Meg) and Na/K ATPase (Na/K; loading control) in renal membrane extracts from adult mice of the respective genotypes.

As well as affecting trafficking of megalin and its co-receptors, the ICD may have been expected to modulate renal gene transcription based on previous studies in cell culture.[14] This hypothesis could not be confirmed in the TgICD model as we failed to detect specific (*megalin, Nhe3*) or global changes in the renal transcriptome.

In conclusion, our studies argue that the physiological functions of megalin require the full-length receptor activity, likely for interaction with ligands in the extracellular space, and that no obvious autonomous functions as yet can be assigned to its soluble tail. Clearly, we cannot exclude effects of the ICD on pathways not tested here or effects that depend on ligand-induced release of the ICD under certain stress conditions (for example, renal protein overload). The generation of this TgICD model represents a major technical advance to test some of these important paradigms in the future.

MATERIALS AND METHODS

Generation of mice

Mice expressing the ICD under control of the *megalin* promoter were produced by homologous recombination in murine embryonic stem cells and subsequent injection of targeted clones into blastocysts. Germ-line transmission of the *TgICD* was tested by PCR and Southern blot (Supplementary Figure S1). Mouse lines from two independent clones were generated, which gave identical results. Biochemical, histo-anatomical, and functional characterizations were performed according to the techniques described in the Supplementary Information.

Primary PTCs

PTCs were prepared as described.[26] Confluent cell layers were incubated with 0.5 mg/ml fluorescein isothiocyanate-conjugated bovine serum albumin for 15 min at 37°C. Thereafter, fluorescence signals were determined in cell lysates and normalized for total protein in the samples.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Targeting of the murine megalin locus.
Figure S2. Immunohistological detection of megalin, ICD, and cubilin in kidneys of *megalin*$^{+/+}$ and *megalin*$^{TgICD/TgICD}$ embryos.
Figure S3. The intracellular domain does not rescue the forebrain defects in megalin-deficient embryos.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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