Mutually dependent localization of megalin and Dab2 in the renal proximal tubule

J. Nagai,1,2 E. I. Christensen,1 S. M. Morris,3 T. E. Willnow,4 J. A. Cooper,3 and R. Nielsen1

1Cell Biology, Institute of Anatomy, University of Aarhus, Aarhus, Denmark; 2Department of Pharmaceutics and Therapeutics, Hiroshima University, Hiroshima, Japan; 3Fred Hutchinson Cancer Research Center, Seattle, Washington; and 4Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany

Submitted 6 August 2004; accepted in final form 25 April 2005

Nagai, J., E. I. Christensen, S. M. Morris, T. E. Willnow, J. A. Cooper, and R. Nielsen. Mutually dependent localization of megalin and Dab2 in the renal proximal tubule. Am J Physiol Renal Physiol 289: F569–F576, 2005. First published May 3, 2005; doi:10.1152/ajprenal.00292.2004.—Disabled-2 (Dab2) is a cytoplasmic adaptor protein that binds to the cytoplasmic tail of the multiligand endocytic receptor megalin, abundantly expressed in renal proximal tubules. Deletion of Dab2 induces a urinary increase in specific plasma proteins such as vitamin D binding protein and retinol binding protein (Morris SM, Tallquist MD, Rock CO, and Cooper JA. EMBO J 21: 1555–1564, 2002). However, the subcellular localization of Dab2 in the renal proximal tubule and its function have not been fully elucidated yet. Here, we report the characterization of Dab2 in the renal proximal tubule. Immunohistocytochemistry revealed colocalization with megalin in coated pits and vesicles but not in dense apical tubules and the brush border. Kidney-specific megalin knock-out almost abolished Dab2 staining, indicating that Dab2 subcellular localization requires megalin in the proximal tubule. Reciprocally, knockout of Dab2 led to a redistribution of megalin from endosomes to microvilli. In addition, there was an overall decrease in levels of megalin protein observed by immunoblotting but no decrease in clathrin or α-adaptor protein levels or in megalin mRNA. In rat yolk sac epithelial BN16 cells, Dab2 was present apically and colocalized with megalin. Introduction of anti-Dab2 antibody into BN16 cells decreased the internalization of 125I-labeled receptor-associated protein, substantiating the role of Dab2 in megalin-mediated endocytosis. The present study shows that Dab2 is localized in the apical endocytic apparatus of the renal proximal tubule and that this localization requires megalin. Furthermore, the study suggests that the urinary loss of megalin ligands observed in Dab2 knockout mice is caused by suboptimal trafficking of megalin, leading to decreased megalin levels.

receptor-mediated endocytosis; kidney; adaptor proteins

RECENT RESEARCH HAS DEMONSTRATED that megalin (35) is one of the most important receptors for protein reabsorption in the renal proximal tubules (reviewed in Refs. 4 and 5). The giant endocytic receptor (600 kDa) is abundantly expressed in the endocytic apparatus of the renal proximal tubule, including the brush border, clathrin-coated pits, clathrin-coated vesicles, and dense apical tubules. Its ligands include Ca2+, vitamin binding proteins, apolipoproteins, hormones, enzymes, and drugs as well as receptor-associated protein (RAP), a chaperone for the low-density lipoprotein receptor (LDLR) gene family (38). Megalin has a large NH2-terminal extracellular region, a single transmembrane domain, and a short COOH-terminal cytoplasmic tail (11, 35). The extracellular region contains four clusters of cysteine-rich, complement-type repeats forming the ligand-binding regions characteristic of the LDLR gene family. The sequence of the cytoplasmic tail has little similarity to that of other members of the LDLR gene family except for the NPXY motifs, which are known to serve as the sorting signal for rapid endocytosis of the LDLR via clathrin-coated pits (2, 11, 35).

Disabled-2 (Dab2) was isolated as a phosphoprotein involved in colony-stimulating factor-1 signal transduction (40). Interestingly, Dab2 expression is downregulated during prostate degeneration and in many human carcinomas and accordingly is also called deleted in ovarian cancer-2 (6, 25, 26, 37). In addition, Dab1, a related protein expressed predominantly in the brain, binds to the NPXY motif(s) in the cytoplasmic tail of very low-density lipoprotein receptor and apolipoprotein E receptor 2 through the phosphotyrosine binding (PTB) domain of Dab1 (9, 12–16, 22). This interaction between the members of the LDLR gene family and Dab1 plays an important role in neuronal positioning (12, 13, 15). Thus some of the members of the LDLR gene family might serve as transmembrane signal transduction molecules (9, 16, 32, 39).

Much attention has been focused on the involvement of the cytoplasmic tail domain of megalin in subcellular localization, endocytic trafficking, and signaling (5, 32–34, 36, 39). The cytoplasmic tail domain of megalin has binding sites for some intracellular protein-protein interaction domains including Src homology 3 (SH3) binding motifs (PPXXP) and NPXY motifs, which bind to SH3 domains and PTB domains, respectively (11, 35). Recently, it has been found that Dab2 binds to the cytoplasmic tail of megalin by using in vitro methods such as coimmunoprecipitation and binding assays (33). Furthermore, Morris et al. (29) observed that the deletion of the Dab2 gene in mice induced an increase in urinary excretion of vitamin D binding protein (DBP) and retinol binding protein (RBP), ligands of megalin (5). These observations may indicate an involvement of Dab2 in megalin-mediated endocytosis in renal proximal tubules under in vivo conditions. However, the precise localization of Dab2 in the renal proximal tubules has not been determined, and the role of Dab2 in megalin-mediated endocytosis has not been fully characterized.

The present study was performed to investigate the subcellular localization of Dab2 in the renal proximal tubules by immunohistocytochemistry and to approach the mechanism underlying increased urinary excretion of megalin ligands in Dab2 knockout mice by investigating Dab2 and megalin levels in knockout mouse models.
MATERIALS AND METHODS

Animals

Dab2 conditional knockout mice were prepared by breeding Dab2<sup>−/−</sup> to Dab2<sup>+/+</sup> Meox2<sup>cre/+</sup>. This circumvents the embryonic lethality caused by Dab2 depletion, and allows the generation of mice lacking Dab2 genes in most cells (29). Young adult Dab2<sup>−/−</sup> and Dab2<sup>+/−</sup> mice were anesthetized, perfused with 4% paraformaldehyde in PBS, and kidneys were further processed (3). The experimental protocol was approved in advance by the Animal Experiments Inspectorate, Ministry of Justice (Denmark).

The generation of mice carrying a kidney-specific megalin gene deletion has been described before (19). Cre recombinase-mediated inactivation of the megalin gene in the kidney results in a complete loss of receptor expression in ~80–90% of all cells in the renal proximal tubule.

Antibodies

Rabbit anti-Dab2 polyclonal antibody (H-110) and goat anti-clathrin heavy chain polyclonal antibody (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-megalin polyclonal antibody and rabbit megalin polyclonal antibody were obtained as described previously (3, 24, 41). Mouse anti-β-adaptin monoclonal antibody was from Affinity BioReagents (Golden, CO). Horseradish peroxidase-conjugated goat anti-rabbit Ig (P448), rabbit anti-sheep Ig (P163), rabbit anti-goat Ig (P449), goat anti-mouse Ig (P447), and TRITC coupled to swine anti-rabbit Ig (R156) were from Dako (Copenhagen, Denmark). Alexa Fluor 488 donkey anti-sheep Ig (A11015) was obtained from Molecular Probes (Leiden, the Netherlands).

Immunocytochemistry

For light microscopy, semithin (0.8 μm) cryosections were cut at −80°C on an FCS Reichert Ultracut S cryomicrotome (Reichert-Jung, Vienna, Austria) and placed on gelatin-coated glass slides. The sections were incubated with rabbit anti-Dab2 antibody (1:20–1:80) or sheep anti-megalin antibody (1:20,000) at room temperature for 1 h following preincubation in 10 mM PBS containing 50 mM glycine and 0.1% skim milk. After incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies, the peroxidase was visualized with diaminobenzidine, and sections were counterstained with Meier’s hematoxylin stain and examined in a Leica DMR electron microscope equipped with a Sony 3CCD color video camera and a Sony Digital Still recorder (Sony, Tokyo, Japan). For electron microscopy, ultrathin (70–90 nm) cryosections were obtained, and the sections were incubated with rabbit anti-Dab2 antibody (1:80–1:240) and/or sheep anti-megalin antibody (1:20,000) followed by incubation with gold-conjugated secondary antibodies (British BioCell, Cardiff, UK). The cryosections were embedded in 2% methylcellulose containing 0.3% uranyl acetate and were examined in a Phillips CM 100 electron microscope.

Morphometric Determination of Megalin Localization in Kidneys from Normal and Dab2 Knockout Mice

Sections from control and Dab2 knockout mice were prepared for electron microscopy as described above. The sections were stained with sheep anti-megalin antibody 1:20,000 and developed with a secondary antibody conjugated to 10-nm gold particles. Eight randomized pictures were taken from six control and six knockout mice at a magnification of ×25,000. All pictures were from tubules that were sectioned in the longitudinal axis of the microvilli. Areas encompassing the microvilli and cytoplasm were measured and gold particles were counted by the use of AnalySIS.

Immunoblotting

Crude kidney membranes for immunoblotting were prepared as described previously (1, 21). Briefly, the excised kidneys were homogenized in an ice-cold buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2) for 30 s with an IKA Ultra-Turrax T8 homogenizer (IKA Labortenik). The homogenate was centrifuged at 4,000 g for 15 min at 4°C. The supernatant was transferred to an Ultra-Clear tube (Beckman Instruments, Fullerton, CA) and centrifuged at 17,000 g for 30 min at 4°C in an L8–70 M ultracentrifuge (Beckman Instruments) with rotor 70.1 T1. The pellet was suspended in the ice-cold buffer used for the homogenization and was heated for 5 min at 95°C in Laemmli sample buffer containing 25% SDS and 1% 2-mercaptoethanol. The samples (5 μg of protein) were run on 3–16% SDS polyacrylamide gradient gels and then transferred to a nitrocellulose membrane for 60 min at 4°C. Subsequently, the membrane was blocked in 5% skim milk in PBS (PBS-T; 80 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h. The membranes were washed for 15 min in PBS-T followed by washing twice for 5 min and incubated overnight at 4°C with primary antibody in PBS-T with 1% BSA [rabbit anti-Dab2 antibody (1:100), rabbit anti-rat megalin antibody (1:1,000), goat anti-clathrin heavy chain antibody (1:400), or mouse anti-β-adaptin antibody (1:100)]. After washing for 15 min in PBS-T followed by washing twice for 5 min, the blots were incubated for 1 h with the horseradish peroxidase-conjugated secondary antibody, washed three times in PBS-T, and visualized with enhanced chemiluminescence (Amersham International, Buckinghamshire, UK).

Cell Culture

Rat yolk sac carcinoma (BN16) cells were cultured as described previously (7, 20). Briefly, BN16 cells were grown in 25-cm<sup>2</sup> plastic culture flasks (Corning Costar, Badhoevedorp, Holland) in Eagle’s Minimal Essential Medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Biological Industries, Fredensborg, Denmark), 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Bio-Whittaker) in a humidified atmosphere of 5% CO<sub>2</sub>-95% air at 37°C. The cells were subcultured every fourth day with a split ratio of 1:5 by using 0.02% EDTA and 0.05% trypsin (Bio-Whittaker). Experiments were carried out with confluent monolayers of BN16 cells cultured in 24-well plates (Nagle Nunc International) for quantitative uptake studies.

Immunofluorescence Microscopy

Co-localization of Dab and megalin. Immunohistochemistry was performed on cells grown confluent in flasks, fixed in 2% paraformaldehyde in 0.1 M cacodylate buffer, washed three times in cacodylate buffer, and scraped off in 1% gelatin in cacodylate buffer at 37°C. The cells were centrifuged twice in 1% gelatin, and finally a few drops of 12% gelatin were added. The gelatin containing the cells was cooled on ice and infiltrated with 2.3 M sucrose in 0.01 M PBS, pH 7.4, for 2 h. The blocks were frozen on nails. Sections were made and labeled with a mixture of primary antibodies, rabbit polyclonal anti-Dab2 1:10 (H110) and sheep polyclonal anti-megalin 1:4,000, and then incubated with a mixture of secondary antibodies, TRITC coupled to swine anti-rabbit Ig 1:20 and Alexa Fluor 488 donkey anti-sheep IgG (H+L) 1:300.

Effect of anti-Dab2 antibody transfection on iodinated RAP uptake. Anti-Dab2 antibody was transfected into BN16 cells with the BioPorter protein delivery reagent from BioCarta. Briefly, BN16 cells cultured on 24-well plate for 3 days were incubated with the protein delivery reagent (5 μl) containing rabbit polyclonal anti-Dab2 antibody (H-110, 1 μg of protein) in a 5% CO<sub>2</sub> incubator at 37°C for 1 h. As a control, the same amount of rabbit Ig (X903, Dako) was used. After being washed three times, the cells were preincubated with

AJP-Renal Physiol • VOL 289 • SEPTEMBER 2005 • www.ajprenal.org
serum-free EMEM containing 0.5% ovalbumin for 10 min at 37°C and then were incubated with the above medium including iodinated RAP for 30 min in the 5% CO₂ incubator. At the end of the incubation, the uptake buffer was removed and 200 μl of 0.1 M NaOH were added to dissolve the cells. The amount of the ligand taken up by the cells was measured by counting the radioactivity. The difference between the two groups was determined by a Student’s t-test. Protein was determined by a Bio-Rad Protein Assay with bovine serum albumin as the standard.

Northern Blotting

Digoxigenin (Dig)-labeled RNA probes for megalin and actin were produced by RT-PCR and in vitro transcription.

RT. Total RNA was purified from mouse kidney cortex with TRIzol reagent (Invitrogen). Purified RNA (0.1 μg) was incubated in a RT reaction mixture for 30 min at 42°C in a Genius thermocycler (Techne).

PCR. PCR was performed with a Hotstartaq master mix kit (Qiagen), 1 μl of RT, and 10 pmol of each primer. The identity of the products was confirmed by Big Dye termination sequencing and analyzed on an ABI PRISM 310 Genetic Analyzer (PerkinElmer). Megalin primers had the following sequences and localization in the rat sequence (gi 561852): sense 5’-cagcagtgactgtaccagaa-3’, position 13762–13781; and antisense 5’-tgagtgcttaaatgtc-3’, position 14141–14122, giving a 380-bp product. PCR was conducted with an annealing temperature of 60°C. Actin primers had the following sequences and localization in the rat sequence (gi 57573): sense 5’-taagcattataagccgac-3’, position 180–199; and antisense 5’-tacgtgggtgatgaggccca 3’, position 529–510, giving a 350-bp product. PCR was conducted with an annealing temperature of 65°C. PCR products containing the T7 promoter sequence were produced from the purified PCR products and the primers described above except that the sense primer included a T7 sequence in the 5’-end. The T7 DNA was used for in vitro transcription in a mixture containing Dig-11-UTP (Roche) to produce the Dig-labeled RNA probes.

Blotting. 10 μg of total RNA was separated on a 1% agarose-2% formaldehyde gel. The loaded amount of RNA was adjusted according to the optical density of the purified RNA. After electrophoresis, the integrity of the RNA was confirmed and the RNA was transferred to a nylon membrane (Roche) by capillary blotting, and finally the RNA was linked to the membrane by UV linking (Hoefer UVC 500). The blots were prehybridized and hybridized with Dig-labeled RNA probes (megalin 20–24 μg, actin 8 μg) overnight at 68°C. Afterward, the blots were washed and blocked. Anti-digoxin-AP conjugate (1:10,000, Sigma) was applied and incubated with CSPD (Roche). The developed bands were quantified by the Fluor-S MultiImager system (Bio-Rad). The intensity of the actin bands was used to standardize the megalin bands.

RESULTS

Localization of Dab2 in the Endocytic Apparatus of the Renal Proximal Tubule

In kidneys from Dab2 knockout mice, Dab2 labeling could not be detected in proximal tubules by light microscopic immunohistochemistry, confirming the complete loss of Dab2 and the specificity of the antibody used for the detection of Dab2 (Fig. 1A). Kidneys from control mice showed a band of Dab2 labeling below the brush border, and labeling of endocytic vacuoles was observed in the cytoplasm of the renal proximal tubules from control mice (Fig. 1B). Electron microscopic immunocytochemistry of cryosections from control mice confirmed labeling of the endocytic apparatus. More specifically, labeling was detected in apical coated pits and apical vesicles but virtually not in dense apical tubules (defined as membrane limited longitudinal structures with diameters slightly smaller than the diameter of a microvillus) (Fig. 2).

Absence of Dab2 in Megalin-Deficient Cells from Kidney-Specific Megalin Knockout Mice

Kidney-specific megalin knockout mice lack megalin in most but not all kidney cells (19). Investigation of Dab2 in kidney sections from these mice by fluorescence microscopy revealed Dab2 only in cells that still expressed megalin (Fig. 3). Most of the proximal tubule cells apparently lacked both megalin and Dab2. Immunoelectron microscopy for megalin and Dab2 also demonstrated that Dab2 labeling was virtually absent in megalin-deficient cells (Fig. 4). In the renal proximal tubular cells that did express megalin, Dab2 colocalized with megalin in parts of the apical endocytic apparatus such as coated pits and small endocytic vacuoles but was almost undetectable in dense apical tubules, where megalin is present.

Fig. 1. Immunohistochemistry using anti-disabled-2 (Dab2) antibody on cryosections of kidney cortex from a Dab2 knockout mouse (A) and a control mouse (B). Magnification: ×1,500.
The reduced staining of Dab2 in megalin–/– cells could be due to a decrease in Dab2 protein levels or due to decreased sensitivity of detection owing to diffuse localization within the cell. These possibilities could not be conclusively distinguished because of the mosaic nature of the kidney-specific knockout. However, immunoblotting of kidney homogenates from mice with megalin knockout in all cells did not reveal any decrease in Dab2 compared with controls (result not shown). This suggests that Dab2 accumulation in apical endocytic pits and vesicles requires megalin.

**Decreased Megalin Levels in Dab2 Knockout Mice**

Immunohistocytochemistry for megalin in the proximal tubule from Dab2 knockout mice was examined and compared with megalin in control mouse kidney. As shown in Fig. 5, there was apparently a decrease in the subcellular compartments containing megalin of the renal proximal tubule in addition to an apparent reduction in the number of coated pits and coated vesicles, as described previously (29). Decreased levels of megalin in the apical cytoplasm were supported by morphometric analysis of electron microscopic sections (110 gold particles/μm² in controls vs. 72 gold particles/μm² in knockouts, Table 1). Furthermore, this analysis showed that megalin levels were increased in the microvilli (96 gold particles/μm² and 150 gold particles/μm² in controls and knockouts, respectively, Table 1). This suggests a redistribution of megalin from vesicles of the apical cytoplasm to microvilli, consistent with decreased endocytosis, when Dab2 is absent.

Surprisingly, immunoblotting of crude membranes from Dab2 knockout mice and control mice revealed an overall...
decrease in megalin levels when Dab2 was absent (Fig. 6). In contrast, no obvious differences in protein levels of clathrin and α-adaptin, the α-subunit of clathrin adaptor protein AP-2, were found between Dab2 knockout mice and control mice. As expected, Dab2 was not detected in kidney from knockout mice. Northern blot analysis did not reveal any differences in megalin mRNA expression between kidneys from Dab2 knockout mice and control mice (Fig. 7).

This suggests that decreases in megalin protein levels are due to a decreased rate of protein synthesis or increased megalin protein turnover.

**Involvement of Dab2 in Megalin-Mediated Endocytosis**

To examine the role of Dab2 in receptor-mediated endocytosis, uptake studies were performed in BN16 cells, a cell line derived from the rat yolk sac highly expressing megalin. First, we performed double labeling immunofluorescence on confluent BN16 cell monolayers with anti-Dab2 antibody and anti-megalin antibody. The labeling pattern for Dab2 was present apically and partly colocalized with megalin (Fig. 8). Next, we introduced anti-Dab2 antibody into the confluent BN16 cell monolayers to inhibit the function of Dab2, and then uptake and binding of iodinated RAP in the cells was measured (Fig. 9). Uptake and binding of 125I-RAP was slightly but significantly reduced in the transfected cells.

**DISCUSSION**

Dab2 is present in various tissues, including heart, lung, liver and skeletal muscle, and is highly abundant in the kidney.
Dab2 has been demonstrated to bind to the cytoplasmic tail of megalin by several approaches, such as two-hybrid screening, protein binding assay, and coimmunoprecipitation (33). Recently, an increase in urinary excretion of specific plasma proteins such as DBP and RBP in Dab2 knockout mice was reported (29). These proteins represent ligands to megalin. Thus Dab2 may play an important role in megalin-mediated endocytosis in the renal proximal tubule. The present study has been performed to clarify the cellular localization of Dab2 in the renal proximal tubule and its role in megalin-mediated endocytosis.

We found that Dab2 is abundantly expressed in parts of the apical endocytic apparatus such as coated pits and coated vesicles, but it is almost absent in the brush border and dense apical tubules of the renal proximal tubules from normal mice. The low Dab2 staining in the brush border is in contrast to immunofluorescence data obtained by Oleinikov et al. (33). The reason for this discrepancy is unknown and difficult to comment on as micrographs were not shown in the paper of Oleinikov et al. However, in fibroblasts and nonpolarized epithelial cells (HeLa), Dab2 localization depends on a region that binds to adaptin and clathrin (23, 27), which are present in coated structures and not associated with the microvillar membrane. Immunoelectron microscopic analysis revealed that labeling for Dab2 was present at the cytoplasmic side of the vesicular membrane of the endocytic apparatus, suggesting that Dab2 serves as a cytoplasmic adaptor protein mediating protein-protein interactions. In addition, colocalization of megalin...
and Dab2 was observed in apical coated pits and vesicles but not in dense apical tubules of the renal proximal tubules. Considering that the cytoplasmic tail of megalin has been shown to interact with Dab2 (33), Dab2 may be involved in megalin-mediated endocytosis in the renal proximal tubular cells by directly interacting with the cytoplasmic tail of megalin during endocytosis, but dissociating from megalin during recycling of the receptor. Similarly, Dab2 was absent from uncoated endosomes in fibroblasts (27).

Analysis of kidney-specific megalin knockout mice, which show a severe impairment in renal absorption of protein owing to a significant decrease in the number of proximal tubular cells expressing megalin (8, 19), revealed that Dab2 localization to the endocytic apparatus is strictly dependent on megalin. An almost complete loss of Dab2 in the endocytic apparatus was observed in megalin-deficient cells of the renal proximal tubule, whereas Dab2 was expressed normally and partly colocalized with megalin in normal tubular cells expressing megalin. These results suggest that the association of Dab2 with coated pits in polarized cells may require cooperative association with the megalin cargo as well as with clathrin and AP-2, whereas in fibroblasts the association with coated pits appears to be independent of cargo (27).

Several recent studies (10, 23, 27) have demonstrated that Dab2 interacts directly with phosphoinositides, clathrin, and the α-adaptin subunit of clathrin AP-2, which are components of clathrin-coated pits and vesicles. The subcellular localization of Dab2 in nonpolarized cells is not due to the PTB domain but to the central region that binds to the cytoplasmic tail of megalin during endocytosis, but dissociating from megalin during recycling of the receptor. Similarly, Dab2 was absent from uncoated endosomes in fibroblasts (27).

Several recent studies (10, 23, 27) have demonstrated that Dab2 interacts directly with phosphoinositides, clathrin, and the α-adaptin subunit of clathrin AP-2, which are components of clathrin-coated pits and vesicles. The subcellular localization of Dab2 in nonpolarized cells is not due to the PTB domain but to the central region that binds to the cytoplasmic tail of megalin during endocytosis, but dissociating from megalin during recycling of the receptor. Similarly, Dab2 was absent from uncoated endosomes in fibroblasts (27).

Megalin protein levels and subcellular localization also depend on Dab2. Electron immunohistochemical analysis showed a decrease in megalin in endosomes, and an increase in megalin in microvilli, in renal proximal tubular cells from Dab2 knockout mice, consistent with a role for Dab2 in megalin endocytosis. Surprisingly, total megalin levels were reduced in Dab2 knockout mice, as observed by immunoblotting. However, there was no significant change in megalin mRNA level between Dab2 knockout mice and control mice, suggesting that the decrease in megalin protein is due to decreased protein synthesis or increased protein turnover. The first and third NPXY motifs in the cytoplasmic tail of megalin are responsible for efficient endocytosis, whereas the second NPXY-like motif is essential for the apical sorting of megalin (36). In addition, the third NPXY motif has been suggested to be involved in the interaction between the PTB domain of Dab2 and the cytoplasmic tail of megalin (33). Therefore, a decrease in megalin levels in Dab2 knockout mice may result from an impaired trafficking of megalin through the endocytic/recycling pathway, leading to increased megalin shedding or degradation.

Even though Dab2 interacts with clathrin and AP-2, and there is a decrease in the number of apical coated pits and apical coated vesicles in the renal proximal tubular cells from Dab2 knockout mice (29) and megalin knockout mice (18), the levels of clathrin and α-adaptin in Dab2 knockout mouse kidneys were normal. Immunohistochemistry for clathrin also revealed equal levels in Dab2 knockout mice and control mice (data not shown).

To investigate the impact of decreased megalin levels induced by Dab2 knockout, we transfected anti-Dab2 rabbit antibodies into BN16 cells. This slightly but significantly decreased internalization of RAP, a high-affinity ligand for megalin, compared with that of control rabbit Ig. This is in accordance with the observations obtained by immunohistochemistry in Dab2 knockout mice, where deletion of Dab2 does not completely abolish endocytosis of RBP and DBP (results not shown).

Dab2 knockout mice present an increase in urinary excretion of DBP and RBP, but the increases are not as severe as in full megalin knockout mice, which exhibit vitamin D deficiency and bone formation defects (31). Indeed, there was a decreased endosomal level of megalin in Dab2 knockout mice, and megalin protein levels were reduced but not completely disrupted by the absence of Dab2. Thus another adaptor protein binding to the cytoplasmic tail of megalin, such as autosomal recessive hypercholesterolemia (30), may substitute for Dab2 in Dab2 knockout mice.

In conclusion, these experiments show the presence of Dab2 in coated structures of the endocytic apparatus of the proximal tubule. Here, its presence seems to be dependent on megalin or other factors associated with megalin. On the other hand, knockout of the Dab2 gene decreases megalin levels and alters the subcellular distribution of megalin. These results indicate that Dab2 and megalin mutually regulate each other’s localization in renal proximal tubule cells.

ACKNOWLEDGMENTS

The authors thank Inger Blenker Kristoffersen, Hanne Sidelmann, Pia Kamuk Nielsen, Anne Merete Hass, and Priscilla Kronstadt O’Brien for excellent technical assistance.

GRANTS

This study was funded by Novo Nordisk, the Karen Elise Jensen Foundation, National Institute of General Medical Sciences Grant GMO-66257, The European Commission (EU, Framework Program 6, EurEGene, contact number 05085), and The Carlsberg Foundation.

REFERENCES


