Megalin and cubilin expression in gallbladder epithelium and regulation by bile acids

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Abstract Cholesterol crystal formation in the gallbladder is a key step in gallstone pathogenesis. Gallbladder epithelial cells might prevent luminal gallstone formation through a poorly understood cholesterol absorption process. Genetic studies in mice have highlighted potential gallstone susceptibility alleles, *Lith* genes, which include the gene for megalin. Megalin, in conjunction with the large peripheral membrane protein cubilin, mediates the endocytosis of numerous ligands, including HDL/apolipoprotein A-I (apoA-I). Although the bile contains apoA-I and several cholesterol-binding megalin ligands, the expression of megalin and cubilin in the gallbladder has not been investigated. Here, we show that both proteins are expressed by human and mouse gallbladder epithelia. In vitro studies using a megalin-expressing cell line showed that lithocholic acid strongly inhibits and cholic and chenodeoxycholic acids increase megalin expression. The effects of bile acids (BAs) were also demonstrated in vivo, analyzing gallbladder levels of megalin and cubilin from mice fed with different BAs. The BA effects could be mediated by the farnesoid X receptor, expressed in the gallbladder. Megalin protein was also strongly increased after feeding a lithogenic diet.🔒 These results indicate a physiological role for megalin and cubilin in the gallbladder and provide support for a role for megalin in gallstone pathogenesis.—Erranz, B., J. F. Miquel, W. S. Argraves, J. L. Barth, F. Pimentel, and M-P. Marzolo. **Megalin and cubilin expression in gallbladder epithelium and regulation by bile acids.** *J. Lipid Res.* 2004. **45:** 2185–2198.

Supplementary key words gallstone disease • apolipoprotein A-I • apolipoprotein J • farnesoid X receptor • vitamin D receptor • biliary cholesterol • gallbladder epithelial cells • *Lith* genes

Cholesterol gallstone disease is a highly prevalent gastrointestinal disorder in Western countries resulting from

alterations in hepatic and biliary cholesterol homeostasis. A number of studies have shown that gallstone formation in humans is a multifactorial phenomenon, including both environmental and genetic factors (1, 2). It is generally accepted that sustained cholesterol supersaturation of bile is responsible for most, but not all, ancillary defects in the hepatobiliary tree. The underlying basis for cholesterol bile supersaturation is not completely understood. Most efforts to understand this process have focused on mechanisms that govern the hepatic secretion of biliary cholesterol, phospholipids, and bile salts (3, 4). The formation of gallstones is also dependent on downstream events that occur in the gallbladder itself, including changes in the physicochemical properties of biliary lipids and gallbladder motility. For example, the gallbladder acts to concentrate bile through mechanisms that involve the adsorption of water and electrolytes (5, 6), which are largely dependent upon Na^+/H^+ exchange at the apical membrane of the gallbladder epithelium (GBE) (7–9). Gallstone formation is also related to an increase in gallbladder secretion of mucin, which correlates with the promotion of crystallization in experimental and human gallstone disease (10–13). Relevant to the production of cholesterol crystals is the rapid aggregation of cholesterol-phospholipid vesicles that occurs in the gallbladder lumen. In this regard, understanding of the mechanisms of gallbladder lipid adsorption, a normal process occurring in the gallbladder (14, 15), is crucial. The gallbladder absorbs large amounts

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of biliary cholesterol and phosphatidylcholine proportional to their molar ratio in the bile. The physiological process of lipid absorption acts to continuously reduce the molar ratios of biliary cholesterol in the gallbladder lumen, thus inhibiting cholesterol crystallization and gallstone formation (16, 17). In spite of the physiological importance of this lipid absorption process, the mechanisms remain unclear.

Megalin and cubilin are large, multiple-ligand receptors expressed on the apical surface of several epithelial tissues, such as the renal proximal tubule (18–20), the small intestine (21), the visceral yolk sac (19, 22, 23), and the male reproductive system (24). Both proteins are structurally different and bind several ligands in common as well as specific ligands (20). Megalin, a member of the low density lipoprotein receptor family, is a type I transmembrane protein, having a relatively large extracellular domain, composed of four clusters of cysteine-rich complement-type/low density lipoprotein receptor class A repeats, which constitute the ligand binding regions, separated by 17 epidermal growth factor-like repeats and eight cysteine-poor spacer domains containing the YWTD motifs involved in the pH-dependent release of ligands in the endosomal compartments (25). Megalin binds several ligands, some related to lipoprotein metabolism, such as apolipoproteins B, E, J/clusterin (apoJ), and lipoprotein lipase, and also binds Ca^{2+} (20). Megalin also has a negative regulatory influence over the activity of proteins such as type 3 Na^+/H^+ exchanger (NHE3) (26, 27). Cubilin is a 460 kDa peripheral membrane glycoprotein also known as the intrinsic factor receptor (28). It has a unique structure with a short N-terminal element followed by 8 epidermal growth factor-like modules and 27 CUB domains (29). Cubilin lacks a membrane-spanning region, but its N-terminal region contains a cell association domain that can mediate interaction with the plasma membrane (30). Cubilin ligands include the intrinsic factor-cobalamin complex, immunoglobulin light chains, albumin, apolipoprotein A-I (apoA-I)/HDL, Ca^{2+}, and megalin (25, 31). The interaction with megalin is particularly important because megalin mediates the internalization of cubilin and its ligands (32). The cooperative relationship between megalin and cubilin is reflected in the fact that in many absorptive epithelial cells both receptors are coexpressed (20, 25).

A genetic basis for cholesterol gallstone formation exists in mouse strains with differential susceptibility to cholelithiasis. *Lith1* and *Lith2* are gene loci that account for the susceptibility of C57L/J inbred mice to develop gallstones (33). The *Lith1* region on chromosome 2 includes the gene for megalin (34), making it a candidate gene associated with lithiasis. Until recently, little attention has been paid to investigating the involvement of megalin in gallstone disease, perhaps owing to the fact that it is not expressed in the liver and there was no evidence that it was expressed in the biliary tree. Here, we establish that both megalin and cubilin are expressed in the gallbladder. Furthermore, we show that megalin expression by gallbladder epithelial cells (GBECs) is regulated by bile acids (BAs) but not by cholesterol, suggesting the

participation of the transcription factor farnesoid X receptor (FXR). These results suggest that bile constituents that are in contact with GBE could regulate the expression of the receptors in the gallbladder, which could have a central role in the development of gallstone disease.

MATERIALS AND METHODS

Reagents

DMEM, F-12, L-glutamine, penicillin-streptomycin, and trypsin were purchased from GIBCO (Life Technologies, Inc., Grand Island, NY). FBS was from Hyclone (South Logan, UT). MEM (α modification), MEM 100× nonessential amino acid solution, MEM 100× vitamin solution, individual protease inhibitors, glutathione-agarose beads, and all chemical reagents, including BAs, were from Sigma Chemical Co. (St. Louis, MO). *Z*-Guggulsterone was from Steraloids, Inc. (Newport, RI), and guggulipid (*Z*-guggulsterone content, 2.5%), with the brand name of Kiol, was from Garden House Labs. Transwell polycarbonate filter units were from Costar (Cambridge, MA). Polyclonal antiserum to recombinant human megalin cytoplasmic domain (anti-MegT) has been previously described (35), as have the mouse monoclonal antibodies to human and rat megalin, 6c5/3c3 and 1H2 (36–38). Rabbit polyclonal antibodies against a recombinant rat cubilin N-terminal region (anti-RC1) (32) and dog gp80 were previously described (39). Mouse monoclonal antibody to actin (clone AC-40) was purchased from Sigma. Polyclonal antiserum to human scavenger receptor class B type I (SR-BI) and β-actin has been described (40). Protein A-Sepharose was from Repligen (Waltham, MA). Kaleidoscope prestained standards were from Bio-Rad (Hercules, CA), peroxidase-labeled antibodies were from Chemicon (Temecula, CA), and the ECL system was from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK). Complete™ protease inhibitor cocktail was from Roche Molecular Biochemicals (Indianapolis, IN). Immobilon-P transfer membranes were from Millipore (Billerica, MA). Taq polymerase was purchased from Promega (Madison, WI). Oligonucleotide primers were obtained from either Gene Link (Hawthorne, NY) or Qiagen (Valencia, CA). The RNeasy Kit was from Qiagen. DNase I, SuperScript II RT, RNase-Out, dNTP mix, DTT, and random primers were from Invitrogen (Carlsbad, CA).

Animals and diets

Male C57BL/6J, BALB/c, and AKR/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals, 8–10 weeks old, were housed in a humidity- and temperature-controlled room with reverse-cycle lighting and maintained on a water and chow diet [<0.02% (w/w) cholesterol; Prolab RMH3000; PMI Feeds, Inc., St. Louis, MO] ad libitum to allow them to adapt to the environment for at least 2 weeks before experimental feedings. After this period, male C57BL/6J mice were fed a chow diet for 2 weeks and then switched to a chow diet supplemented with BAs [cholic acid (CA) and chenodeoxycholic acid (CDCA) at 0.5% by weight] for 10 days, or to a lithogenic diet (1.25% cholesterol, 15% total fat, and 0.5% CA; TD90221; Harlan Teklad, Madison, WI), or to a 2% cholesterol diet, for 10 days. BAs were added in ethanol to the powdered chow and dried for 24 h. A second group of mice were maintained on a chow diet and given daily treatments, lithocholic acid (LCA; 8 mg/day) or vehicle (corn oil), via gavage for 4 days. An additional group of animals were fed a chow diet containing either 2.5 mg/day *Z*-guggulsterone or 25 mg/day guggulipid for 5 days. Tissues were harvested 24 h after the last treatment as described (41). Animal protocols were carried out according to accepted criteria for the

humane care of experimental animals and approved by the Review Board for Animal Studies of our institution.

Mouse tissue isolation

Tissue specimens were obtained from 8–10 week old mice. To obtain the gallbladder, the cystic duct was first ligated and the gallbladder removed by dissection. Bile was aspirated from the gallbladder using a syringe and stored at −20°C for posterior biliary lipid analysis. The gallbladder was incised longitudinally, washed in PBS, rapidly frozen in liquid nitrogen, and stored at −80°C. Kidney and liver specimens were also isolated, washed in PBS, and stored in a similar way.

Human tissue samples and isolation of GBECs

Human gallbladder tissue was obtained from patients undergoing elective laparoscopic cholecystectomy essentially as described before (40, 42). Normal kidney and liver tissues were obtained from patients subjected to nephrectomy as a result of renal cell carcinoma and partial liver resection as a result of liver cyst, respectively. Quadriceps muscle samples were obtained from patients who underwent orthopedic procedures. Portions of the freshly excised tissue specimens were frozen in liquid nitrogen and stored at −80°C for further use, and the remaining portions were fixed in formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin using standard histological procedures. Freshly excised gallbladder specimens were also used to isolate GBE as described (40). Briefly, freshly excised gallbladder was maintained in cold sterile medium (1:1 DMEM-Ham's F12) and then everted. The mucosa was rinsed with medium and wiped with gauze several times to remove mucus and adherent bile. The gallbladder tunica mucosa was then placed in 0.125% collagenase solution for 20 min at 37°C. Every 5 min, the mucosa was abraded thoroughly using a scalpel and flushed with DMEM. The resulting cell suspension was subjected to centrifugation at 85 *g* for 5 min at 20°C. Microscopic examination of an aliquot of freshly isolated GBECs revealed that more then 95% of cells had epithelial features. GBECs were frozen in liquid nitrogen and kept at −80°C for subsequent protein and RNA extraction, as described below. Studies with human tissues were approved by the Ethics Committee of the Faculty of Medicine, Pontificia Universidad Católica de Chile, and informed consent was given by the individual patients.

Immunohistochemistry

Human gallbladder and kidney tissue specimens were fixed and paraffin embedded. Tissue sections were deparaffinized and rehydrated by standard methods, and endogenous peroxidases were quenched with 0.3% H_2O_2 in methanol for 30 min at room temperature. Immunostaining was performed in a Nexes IHC staining system (Ventana Medical System, Inc., Tucson AZ) essentially as described (40). Incubation with the primary antibody was carried out for 90 min (for megalin, anti-MegT 1:1,200; for cubilin, anti-RC1 1:100; for actin, anti-β-actin 1:100). Bound IgGs were detected using biotinylated secondary antibody and peroxidase-conjugated avidin. Peroxidase activity was revealed with 3,3′-diaminobenzidine hydrochloride and H_2O_2. Subsequently, sections were counterstained with hematoxylin and eosin, dehydrated, cleared, and mounted in Permount. As negative controls, tissue sections were stained as above but using irrelevant isotypic immunoglobulins or without the inclusion of the primary antibody.

RT-PCR analysis of megalin and cubilin expression

Total RNA was prepared from each tissue with the RNeasy mini kit (Qiagen) or alternatively by the guanidinium thiocyanate-phenol chloroform method (43). The RNA was treated with RNase-free DNase I to digest contaminating DNA. First-strand cDNA reactions were performed in a total volume of 20 μl with 1.5 μg of total RNA, 300 ng of random primers, 0.5 mM dNTP mix, 40 units of RNaseOut, 10 mM DTT, and 200 units of Super-Script II RT according to the manufacturer's instructions. As negative controls, each RNA sample was subjected to first-strand cDNA reaction with all components but lacking SuperScript II RT, or reactions were performed using all components except RNA template. After the first-strand cDNA reaction, 1/10th of the cDNA was used as a template for PCR. Each PCR procedure was performed in 50 μl containing 10 pmol of each primer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, and 0.4 units of Taq DNA polymerase. The thermal cycling profile consisted of 95°C for 5 min of initial denaturation, 35 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 45 s, and finally 72°C for 5 min of final extension. A set of specific primers was designed for human and mouse megalin, cubilin, and GAPDH as positive controls. Human primers were as follows. For megalin: hMeg 5′, 5′-TAAGTCAGTGCCC-AACCTTT-3′ (residues 13085–13104), and hMeg 3′, 5′-GCGGT-TGTTCCTGGAG-3′ (residues 13360–13375), GenBank accession number NM_004525. For cubilin: hCub 5′, 5′-GCGGCTTCACT-GCTTCCTA- 3′ (residues 7693–7711), and hCub 3′, 5′-GAGTGA-TGGTGTGCCCTTGT-3′ (residues 8192–8211), GenBank accession number AF034611. For GAPDH: hGAPDH 5′, 5′-GGACCTGAC-CTGCC-3′ (residues 804–817), and hGAPDH 3′, 5′-TACTCCTTG-GAGGC-3′ (residues 1069–1082), GenBank accession number NM_002046. Mouse primers were as follows. For megalin: mMeg 5′, 5′-CCTTGCCAAACCCTCTGAAAAT-3′ (residues 9357–9378), and mMeg 3′, 5′-CACAAGGTTTGCGGTGTCTTTA-3′ (residues 9897–9918), GenBank accession number XM_130308. For cubilin: mCub 5′, 5′-CAACATGGAACACAAACACTTT-3′ (residues 4397–4418), and mCub 3′, 5′-AGCTATTGAATGTACGTCCACA-3′ (residues 4768–4789), GenBank accession number XM_130038. For GAPDH: mGAPDH 5′, 5′-CGGTGTGAACGGATTTGGC-3′ (residues 58–76), and mGAPDH 3′, 5′-GCAGTGATGGCATGGACTGT-3′ (residues 569–588), GenBank accession number NM_008084. For FXR: mFXR 5′, 5′-GCAACTGCGTGATGGACATGT-3′ (residues 743–763), and mFXR 3′, 5′-GCGTACTCCTCCTGAGTCATT-3′ (residues 1386–1406), GenBank accession number U09417. PCR products separated on a 1% agarose gel containing ethidium bromide were visualized and analyzed with the Gel Doc 2000 Gel Documentation System and Quantity One version 4 (Bio-Rad, Hercules, CA). RT and PCR procedures were performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA).

Immunoblotting

Crude membrane fractions were prepared from human GBE, liver, and kidney tissues as well as from pools of murine gallbladders and kidney specimens as described (40). To assay for gallbladder levels of megalin in control and treated mice, glutathione *S*-transferase-receptor-associated protein (GST-RAP) pull downs were performed (see below). Each sample was subjected to non-reducing SDS-PAGE on 6% gels for megalin and cubilin and 7.5% gels for SR-BI and transferred to polyvinylidene difluoride membranes. Membranes were incubated with anti-human megalin 6c5/3c3 (2 μg/ml) for human megalin detection, anti-rat megalin monoclonal antibody 1H2 (2 μg/ml) for GST-RAP pull down samples, or anti-cubilin RC1 (2 μg/ml) or anti-SR-BI (1:2,500) followed by incubations with horseradish peroxidase-conjugated secondary IgGs for 120 min at room temperature. Extracts of LLC-PK1 cells were subjected to reducing SDS-PAGE on 6% gels or 12.5% gels for the detection of megalin or actin, respectively, using anti-MegT (1:4,000) and anti-actin (1:2,500) in the immunoblot. Immunodetection was carried out using the ECL system. Densitometric analysis was performed using the Gel Doc 2000 Gel Documentation System and Quantity One version 4 (Bio-Rad).

The signal generated by anti-actin immunolabeling was used for normalization.

GST-RAP pull down

Recombinant GST-human RAP fusion protein was prepared as previously described (44). Pooled murine gallbladders from control and treated mice were homogenized on ice in homogenization buffer [PBS, 0.5% (v/v) Triton X-100, 2 mM PMSF, pepstatin, antipain, and Complete™ protease inhibitor cocktail] using a Teflon pestle connected to an electrical rotor. Nuclear debris was removed by centrifugation at 3,000 g for 10 min at 4°C, and the supernatants were saved. Protein concentration was determined using a Bradford assay. Equal amounts of protein from the gallbladder extracts (500 μg) were incubated with 80 μl of glutathione-agarose [diluted 1:1 (v/v) beads to buffer] and 30 μg of GST-RAP fusion protein for 8 h at 4°C. The beads were washed four times in homogenization buffer, and nonreducing SDS buffer sample was added to the drained beads. Bound proteins were separated on 6% SDS-PAGE, and megalin was detected by immunoblotting.

Cell lines

MDCK cells (strain II) were maintained in DMEM (Life Technologies) supplemented with 7.5% FBS (Hyclone). LLC-PK1 cells were grown in αMEM (Life Technologies), 10% FBS, and 2 mM glutamine. Brown Norway rat yolk sac cells (BN) (45) were grown in αMEM supplemented with 1% nonessential amino acids and 10% FBS. A canine GBEC line (cGBEC) was obtained from Dr. Sum P. Lee (46) and grown in MEM supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES, 1% MEM nonessential amino acids, and 1% MEM vitamin solution. All culture media were supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin sulfate, and cells were maintained at 37°C in 5% CO_2.

Cell treatments with BAs

LLC-PK1 cells were plated at a density of 65×10^3 cells/cm^2 and 12 h after seeding were treated with different bile salts. Stocks of LCA, CDCA, and CA were prepared in methanol-water (1:1, v/v) adding NaOH when necessary to form the salt. BAs were added to the medium at various concentrations (10–100 μM) and for different lengths of time (24–96 h) as indicated in each experiment. For all of the conditions, the ratio between BA and carrier/medium was 1:1,000 (v/v). During the treatment, the medium was changed every 12 h. At the end of the treatment, the cells were washed three times in PBS and the excess liquid was drained. Subsequently, the cells were scraped in the lysis buffer (PBS containing 1% Triton X-100, 2 mM PMSF, 100 μM pepstatin, antipain, and Complete™ protease inhibitor cocktail) and lysed by passage through a 29 gauge needle eight times. Nuclear debris was removed by centrifugation at 22,000 g for 5 min at 4°C, and the supernatants were saved. The protein content was determined using a Bradford assay, and equal amounts of total protein from each extract were subjected to SDS-PAGE under reducing conditions.

Immunoprecipitation of metabolically labeled megalin and apoJ/clusterin

MDCK and cGBEC cells were plated on 3 cm plates. Additionally, cGBECs were seeded onto 24 mm Transwell polycarbonate filter units (0.4 μm pore size) until the transepithelial resistance reached 1,500 Ω/cm^2, as measured with an EVOM electrometer (World Precision Instruments, Sarasota, FL). Cells were incubated twice with depletion medium (DMEM without methionine and cysteine) and then pulsed with 150 μCi of [^{35}S]methionine/cysteine (New England Nuclear) for 30 min. After 4 h of chase in complete medium containing 10-fold higher levels of methionine (3 mg/ml) and cysteine (6.5 mg/ml), the medium was collected and immunoprecipitations were performed using poly-

clonal antiserum against apoJ as described (39). The cells were lysed in PBS and 1% Triton X-100 with protease inhibitors, and the lysate was subjected to immunoprecipitation using anti-megalin 1H2 antibody. Immune complexes were then precipitated with protein A-Sepharose for 2 h at 4°C. After washing, the precipitated proteins were resolved by SDS-PAGE on 6% gels for megalin and 10% gels for apoJ and the gels were subjected to autoradiography.

Lipid analysis

Biliary BAs were quantified by the 3α-hydroxysteroid dehydrogenase method, lecithin by the inorganic phosphorous procedure, and cholesterol by the cholesterol oxidase assay, as described previously (47). The cholesterol saturation index (CSI) was calculated in accordance with Carey's critical table (48). Cholesterol crystals in gallbladder bile were visualized with a polarized light microscope.

Statistical analysis

Values are expressed as means ± SD. The significance of differences between means was evaluated using an unpaired Student's t-test. The level of significance was set at $P \leq 0.05$.

RESULTS

Expression of megalin and cubilin mRNAs in the mouse and human gallbladder

Using RT-PCR, megalin and cubilin transcripts were detected in gallbladder RNA preparations isolated from three mouse strains, C57BL/6J, AKR/J (**Fig. 1A**), and BALB/c (data not shown). RNA from kidney was used as a positive control, and because liver does not express megalin (49, 50), liver RNA was used as a negative control. We also found that both megalin and cubilin mRNAs were expressed by GBECs isolated from human gallbladder (Fig. 1B). Similar results were obtained using RNA isolated from epithelial cells from gallbladders of other patients (data not shown). RNA derived from human kidney was used as a positive control for the detection of megalin and cubilin. For a negative control, a sample of human skeletal muscle was used. Taken together, these findings are the first indications that megalin and cubilin mRNAs are expressed in the gallbladder.

Immunohistological staining reveals megalin and cubilin expression in human GBE

Immunohistochemistry was used to evaluate the expression and subcellular localization of megalin and cubilin proteins in gallbladder. As shown in **Fig. 2A**, the simple columnar epithelial cells that line the gallbladder prominently express both proteins. Immunostaining for both proteins was apparent throughout the epithelial cells, with higher levels of stain evident in the apical aspects of the cells. In addition, there was an intracellular vesicular pattern of staining consistent with the localization of both proteins within vesicles of the exocytic secretory pathway and/or the endocytic/transcytotic pathway (Fig. 2B). Little or no expression of these proteins was detected in the underlying lamina propria of areolar tissue. No staining was obtained when the primary antibodies were omitted or when irrelevant immunoglobulins were used (data not shown).
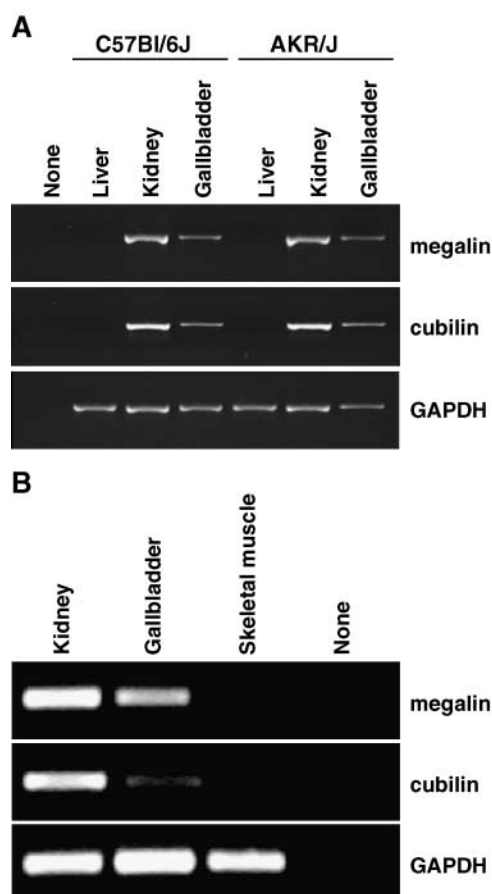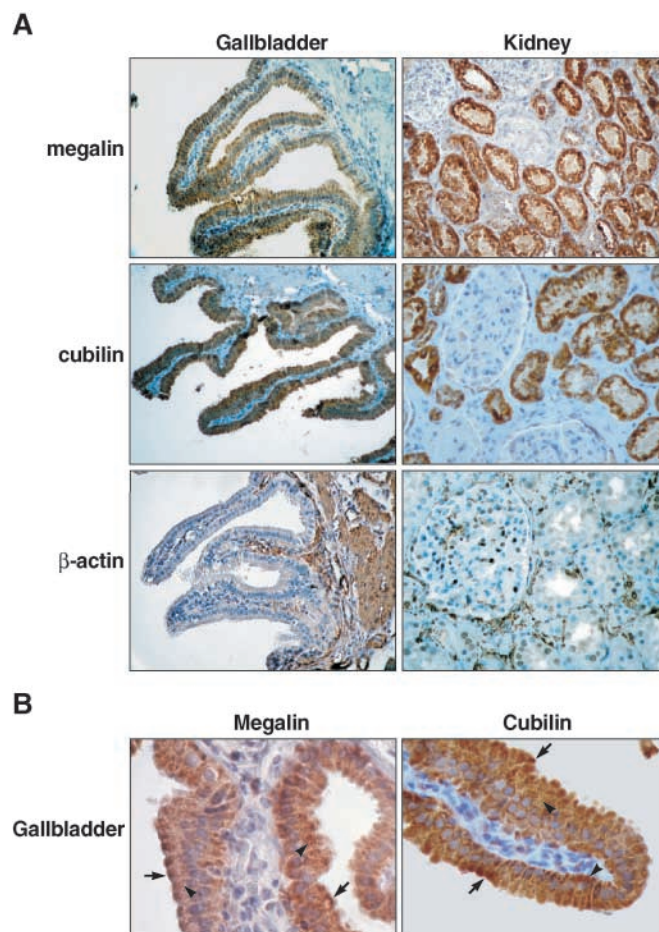
Fig. 1. Megalin and cubilin mRNAs are expressed in the gallbladder. A: RT-PCR analysis of megalin and cubilin transcript expression in the murine gallbladder, liver, and kidney. B: RT-PCR analysis of cubilin and megalin mRNA in human gallbladder, kidney, and skeletal muscle.

Fig. 2. Immunolocalization of megalin and cubilin in human gallbladder. A: Antibodies to megalin (upper row), cubilin (middle row), and β-actin (lower row) were used to label sections of human gallbladder and kidney. Strong immunoperoxidase reactions for both megalin and cubilin are apparent in the gallbladder, localized predominantly in the simple columnar epithelium. No expression of either receptor is observed in the lamina propria. Cubilin and megalin immunolabeling in epithelial cells of proximal renal tubules are shown as positive controls. B: Higher magnification views show that the peroxidase staining for both receptors is throughout the epithelial cells. Apical regions appear to have higher levels of the stain (arrows), but stain is also apparent in basolateral regions and in the punctate intracellular pattern, consistent with localization in intracellular vesicles (arrowheads).

Immunoblot analysis of megalin and cubilin expression in the human gallbladder

Immunoblot analysis was performed on total membrane extracts of GBE isolated from two human gallbladders. As shown in **Fig. 3A**, GBE membrane extracts contained polypeptides immunoreactive with megalin monoclonal antibody. These bands had electrophoretic mobilities similar to those of immunoreactive polypeptides present in membrane preparations from yolk sac BN cells and human kidney. The multiple-band megalin profile observed in gallbladder membranes may reflect breakdown of the ~600 kDa megalin polypeptide, as has been previously suggested in other reports (38, 51). As shown in Fig. 3B, full-length cubilin (~460 kDa) as well as immunoreactive fragments were also detectable in membrane preparations of GBECs, but not in membranes from human liver. Cubilin fragments have been previously described in cubilin preparations isolated from the apical brush-border membranes from rat intestine (21).

Canine GBECs express megalin and cubilin

In addition to the gallbladder, we were also interested to know whether megalin and cubilin were expressed by an available canine gallbladder cell line. cGBEC and MDCK cells were metabolically labeled, and megalin was immunoprecipitated from detergent lysates. **Figure 4A** shows that megalin was present in cGBEC but not in MDCK, as we previously described (35), consistent with our gallbladder tissue data. Although we were not able to detect cubilin by immunoprecipitation from the metabolically labeled cGBECs (data not shown), we were able to detect it by immunoblotting in cGBEC membrane extracts (Fig. 4B).

Canine GBECs express and secrete a megalin ligand, apoJ/clusterin

Considering that bile contains several ligands for megalin and cubilin, including apoE and apoA-I, respectively
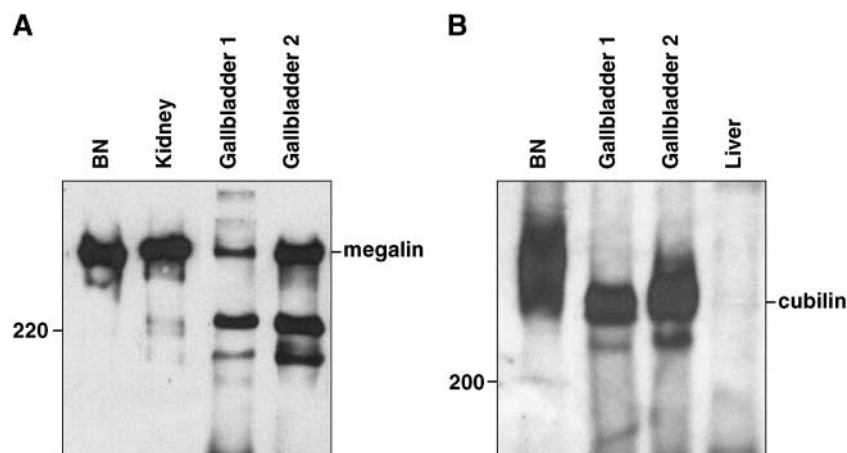
Fig. 3. Immunoblot detection of megalin and cubilin in human gallbladder. Total membranes were prepared from two isolated human gallbladder epithelia (GBEs; 100 μg), human liver (120 μg), human kidney (1.5 μg), and from the rat yolk sac cell line BN (3 μg) and resolved by nonreducing 6% SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes and immunodetection with antibodies to megalin (A) or cubilin (B). Both proteins are present in gallbladder-derived membranes and are detected at sizes corresponding to the full-length and smaller protein fragments.

(52), we were interested to know if cGBECs in culture express apoJ, another ligand for megalin (53). Like apoA-I and apoE, apoJ displays high affinity for lipid and plays a physiological role in lipid transport (54). Conditioned media from the metabolically labeled cells were used for anti-apoJ immunoprecipitation. As shown in Fig. 4C, apoJ is immunoprecipitable from the conditioned culture medium of cGBEC at a level similar to that found in the medium of MDCK cells, a renal distal tubule-derived cell line. ApoJ secretion by cGBECs grown on filters was predominantly apical, suggesting that in vivo it could become enriched with biliary lipids and internalized by megalin expressed in the apical domain of the GBE (Fig. 4D).

Regulation of megalin protein expression by BAs in vitro

The fact that megalin expression is regulated by retinoic acid and vitamin D suggests the involvement of the transcription factors vitamin D receptor (VDR) and retinoid X receptor (RXR) in its regulation (55). Because the hydrophobic secondary BA LCA binds with high affinity to VDR (41), it is also possible that the expression of megalin could be regulated by BAs. To test this hypothesis, we evaluated the effect of BA treatment on the expression of megalin by LLC-PK1 cells. We found that LCA treatment led to a dose-dependent decrease in the expression of megalin, clearly seen at the LCA concentration of 25 μM (**Fig. 5**). The inhibitory effect of LCA (30 μM) was reversible; within 72 h of LCA withdrawal, the level of megalin expression, compared with control cells, was normalized (**Fig. 6**). The downregulation of megalin expression by LCA was counter to our expectations, owing to the fact that LCA is known to lead to the activation of VDR (41). We therefore speculated that LCA might be acting to regulate megalin expression via binding to FXR, a nuclear BA receptor. To test this hypothesis, we evaluated the capacity of primary BAs, which are known agonists of FXR, to regulate megalin expression. As shown in **Fig. 7**, the FXR agonists CDCA and CA increased the expression of megalin in LLC-PK1 cells. In some cases, we also saw a slight increase in megalin expression by lower LCA doses (10 μM) that could be explained by an activation of VDR, because this nuclear receptor has much more affinity for LCA than does FXR (41). These findings clearly indicate that megalin expression is regulated by BAs.

BAs regulate in vivo megalin expression

To evaluate the in vivo effects of BAs on gallbladder expression of megalin, mice were treated with LCA for 4 days. Immunoblot analysis of gallbladder megalin expression showed that after LCA gavage, megalin expression was reduced by $39 \pm 12\%$ (n = 3) compared with levels in gallbladders of control mice (**Fig. 8A**). By contrast, gallbladder expression of cubilin (the full-length cubilin polypeptide plus the cubilin fragment) was not affected by LCA treatment (Fig. 8B). Mice were then fed with diets containing the primary BAs CA and CDCA, FXR agonists, or the FXR antagonists guggulipid and its active component guggulsterone. After these treatments, megalin expression was greatly increased (Fig. 8C, D), which strongly indicates the participation of the FXR nuclear receptor. This apparently contradictory result obtained with the FXR antagonists, acting as agonists, has been described for bile salt export pump (BSEP) expression (56). Relevant to these results was the finding that by RT-PCR, FXR was highly expressed in mouse gallbladder in all conditions, being lower than the level found in LCA-treated animals (data not shown). Taken together, these results indicate that megalin but not cubilin expression in gallbladder is under the control of BAs present in bile, probably through the action of FXR.

Gallbladder expression of megalin but not cubilin is upregulated after the lithogenic diet

We next evaluated the influence of a high-cholesterol/fat/bile salt lithogenic diet or, separately, cholesterol and CA, on gallbladder expression of megalin. C57BL/6J mice
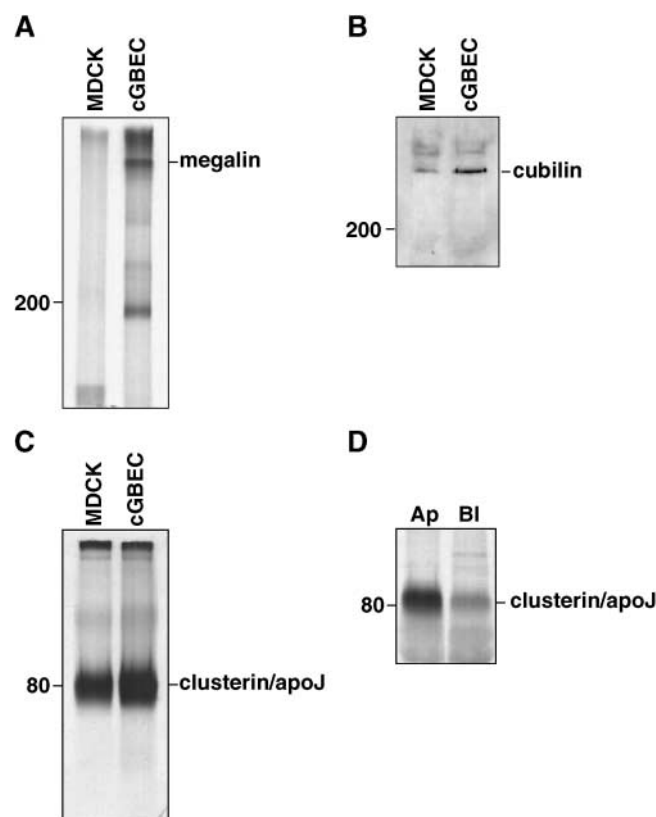
Fig. 4. Canine gallbladder epithelial cells (cGBECs) express megalin and cubilin and secrete apically apolipoprotein J (apoJ), a megalin ligand. A: Megalin in extracts of metabolically labeled canine kidney (MDCK) and canine gallbladder (cGBEC) cells line lysates was assessed by immunoprecipitation using the monoclonal antibody anti-megalin 1H2, and the immunoprecipitates were resolved by 6% SDS-PAGE under nonreducing conditions and subjected to autoradiography. B: Cubilin in 120 μg of membrane extracts of cGBEC and MDCK cells was assessed by immunoblotting after running the samples on 6% SDS-PAGE. C: ApoJ was assessed in the conditioned cultured medium obtained in A by immunoprecipitation using apoJ antibody and autoradiography. D: Filter-grown cGBECs were labeled with [^{35}S]methionine/cysteine, and then the apical (Ap) and basolateral (Bl) media were immunoprecipitated with apoJ antibody. The immunoprecipitates were resolved by 10% SDS-PAGE under nonreducing conditions and subjected to autoradiography.

were fed for 10 days with either a lithogenic diet or a diet supplemented with 2% cholesterol or 0.5% CA. As shown in **Table 1**, gallbladder biliary cholesterol, phospholipids, and CSI were increased significantly (by 4.6-, 1.7-, and 3-fold, respectively) in animals fed the lithogenic diet. As expected, only mice fed the lithogenic diet developed biliary sludge or cholesterol crystals. Animals fed the high-cholesterol diet for 10 days showed a slight but significant ($P < 0.05$) increase in biliary cholesterol content and CSI (1.7- and 1.9-fold, respectively). CA- and LCA-treated animals showed minor changes in biliary lipid levels. Immunoblot analysis was performed on extracts of separate pools of gallbladders from five animals for each condition. As shown in **Fig. 9A**, gallbladder megalin expression increased dramatically in animals fed either a lithogenic
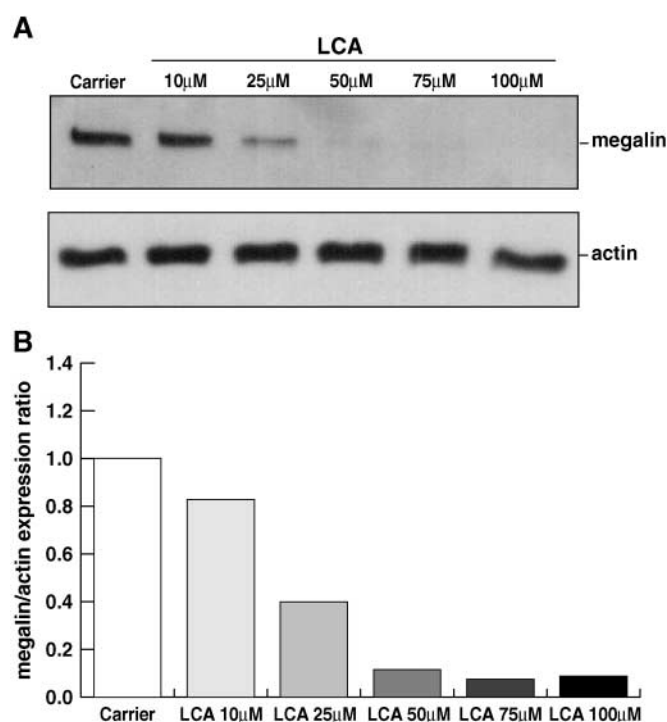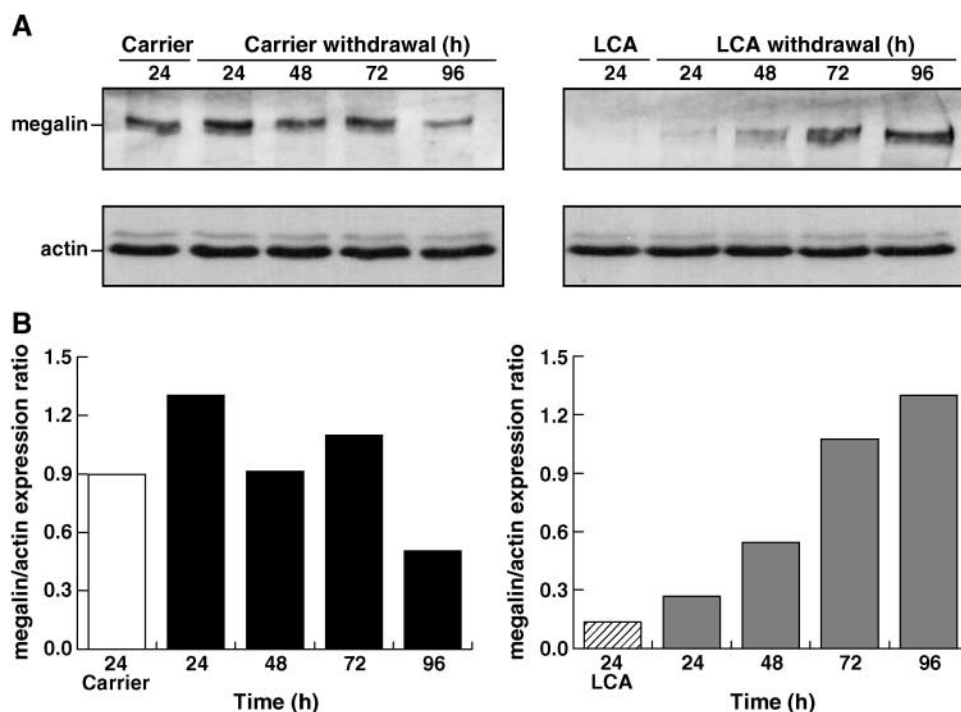

Fig. 5. Regulation of megalin expression by lithocholic acid (LCA) in cultured cells. A: Immunoblot analysis of megalin and actin in LLC-PK1 cells treated with different concentrations of LCA (10–100 μM) or vehicle for 24 h. B: Plots of the megalin/actin ratio based on densitometric analysis of the data shown in A.

diet or a normal chow diet supplemented with CA for 10 days (7.7 ± 0.12-fold and 8.8 ± 0.24-fold, respectively, over controls; n = 2). By contrast, animals fed the high-cholesterol diet (2%) showed no change in gallbladder megalin expression levels. As a control for the effect of the diets, the expression of SR-BI was evaluated because it is already known that this is downregulated after feeding the animals with a lithogenic diet (Fig. 9C) (40). Neither of the above dietary conditions clearly modified cubilin expression (Fig. 9B). Overall, these results indicate that megalin expression in gallbladder is regulated by the composition of BAs present in gallbladder bile, probably acting through a FXR transcription factor. Cholesterol levels seem not to influence megalin expression, as indicated by the lack of correlation between biliary cholesterol concentration, or CSI, and gallbladder megalin expression.

DISCUSSION

In this work, we have shown for the first time that megalin and cubilin, two proteins related to lipid and vitamin metabolism and major endocytic receptors involved in the binding of several ligands, are expressed on the apical surface of the GBE. RT-PCR and immunoblotting established the presence of both megalin and cubilin mRNAs and protein in human and mouse gallbladders. Immunohistochemistry established the presence of both proteins in epithelial cells that line the gallbladder. Megalin, but not

Fig. 6. Downregulation of megalin expression by LCA is reversible. A: Immunoblot analysis of megalin and actin in LLC-PK1 cells cultured with LCA (30 μM) or vehicle for 24 h and then with LCA-free medium for varying periods of time. B: Plot of the megalin/actin ratio based on densitometric analysis of the data shown in A.

cubilin, expression was regulated by BAs, both in vitro and in vivo, suggesting a role for the transcription factor FXR in megalin transcriptional regulation. Furthermore, treatment of mice with different BAs changed megalin protein levels, implying that the BA pool composition of bile could have a role in the regulatio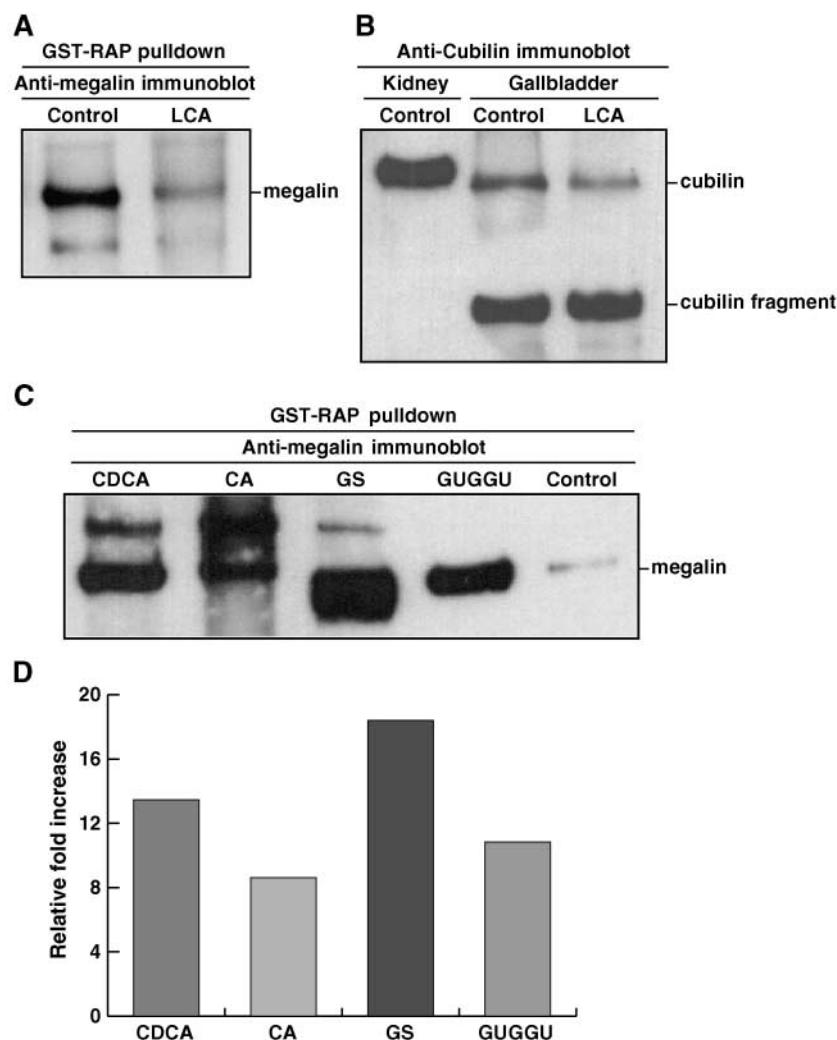n of the receptor expression in gallbladder. Finally, megalin expression was significantly upregulated in gallbladder of mice fed with lithogenic and CA-containing diets but was not affected by a cholesterol-rich diet.

GBECs absorb cholesterol (14, 16, 57), and this activity seems to be impaired in cholesterol gallstone disease (17).
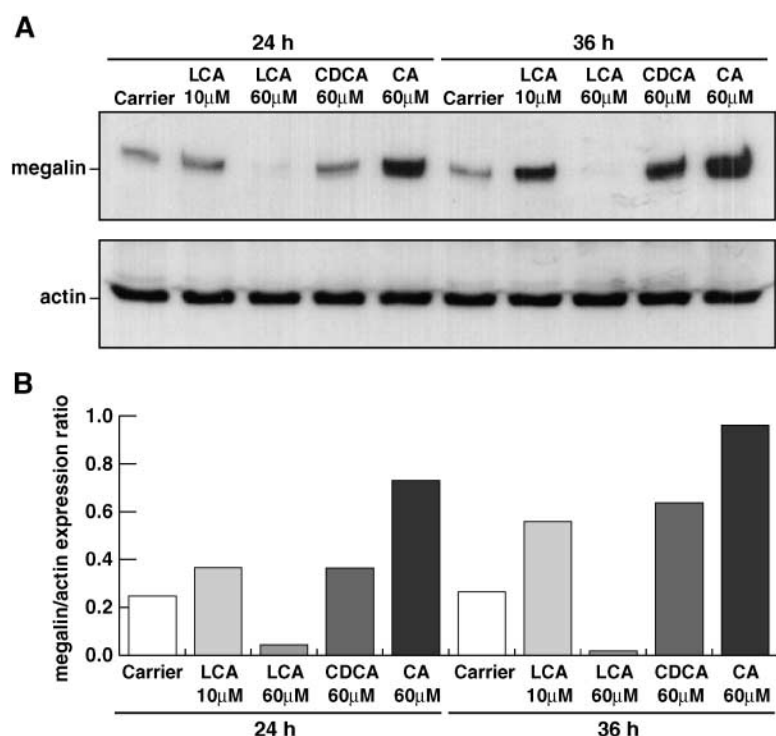
Fig. 7. Megalin protein expression is under the regulation of different bile acids in cell culture. A: Immunoblot analysis of extracts of LLC-PK1 cells cultured with LCA, chenodeoxycholic acid (CDCA), or CA for either 24 or 36 h. B: Plot of the megalin/actin ratio based on densitometric analysis of the data shown in A. LCA had a dual effect, stimulating at a low dose (10 μM) and inhibiting at a high dose (60 μM) megalin protein levels. CDCA and CA stimulated megalin expression at 60 μM.

Fig. 8. Farnesoid X receptor (FXR) ligands regulate megalin but not cubilin gallbladder protein expression in vivo. Megalin (A) and cubilin (B) in gallbladders of LCA-treated and control mice were analyzed by immunoblot. To detect megalin, gallbladder lysates (500 μg of total protein) were subjected to a glutathione S-transferase-receptor-associated protein (GST-RAP) pull down procedure before immunoblot analysis. To detect cubilin, 120 μg of gallbladder total protein was directly analyzed by immunoblotting. Total kidney extract (20 μg of total membranes) was used as a positive control. C: Megalin protein levels in gallbladders of mice fed four different FXR ligands. For each FXR ligand tested, a significant increase in megalin expression was apparent. GS, Z-guggulsterone; GUGGU, guggulipid. D: Densitometric quantification of the immunoreactive megalin polypeptide bands shown in C.

BAs are absorbed by GBECs at a much slower rate compared with cholesterol and phosphatidylcholine. Through such control over BA levels in bile, the gallbladder maintains cholesterol solubility (16). However, it is not known if the cholesterol absorption process is receptor mediated and what is the fate of this cholesterol. Given the role of cubilin and megalin in lipoprotein uptake in other tissues, their apical localization in the GBE supports a possible role in the absorption of cholesterol and perhaps other molecules from the bile.

It has been speculated that biliary apoA-I negatively influences the formation of cholesterol crystals (58). Addition of apoA-I to the luminal side of cultured GBECs enhances cholesterol and phospholipid absorption. ApoA-I binding to these cells is saturable and competitive, suggesting the participation of a receptor (58). Based on the findings presented here, the apoA-I receptor complex formed by megalin and cubilin (32, 59) may play a role in apoA-I uptake by GBECs in the gallbladder. Recent findings indicate that the apoA-I/cholesterol receptor, SR-BI, is also present on the apical surface of GBECs (40, 60). However, no increase in susceptibility to gallstone formation has been observed in SR-BI-deficient mice fed a lithogenic diet (40). Furthermore, there was no change in gallbladder cholesterol content in the SR-BI-deficient mice compared with wild-type animals, suggesting that SR-BI does not have a crucial role in the process of gallbladder cholesterol absorption (40). Other studies have shown the participation of ATP binding cassette (ABC) transporters in the cholesterol efflux from GBECs (57, 61). ABCA1 me-

TABLE 1. Lipid composition of gallbladder bile from C57BL/6 mice fed a control chow diet, a diet supplemented with 2% cholesterol, 0.5% CA, or LCA, and mice gavaged daily with LCA or vehicle

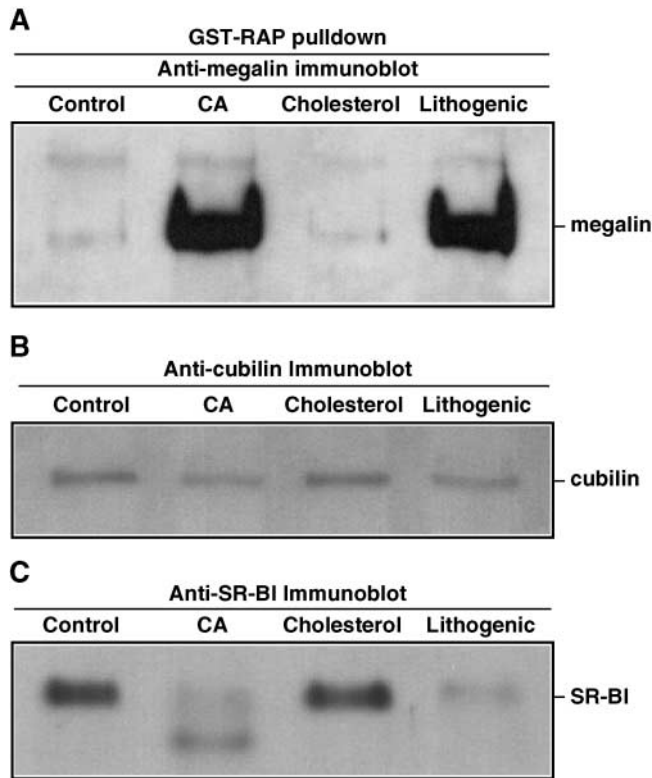| Group (n)	Cholesterol	Phospholipids	Bile Salts	Cholesterol Saturation Index	Cholesterol Crystals or Sludge
	mmol/l				*%*
Control (6)	3.07	14.81	136.7	0.50	0
2% cholesterol (5)	5.41[a]	14.62	116.8	0.96[a]	0
0.5% CA (5)	4.77	14.72	133.9	0.78	0
Lithogenic (6)	13.8[a]	25.9[a]	114.0	1.52[a]	100
Control-LCA (5)	2.92	18.25	186.4	0.35	0
LCA (5)	4.55	19.9	231.0	0.44	0

CA, cholic acid; LCA, lithocholic acid. Male mice (n = 5 or 6 in each group) were fed the different experimental diets for 10 days. LCA (8 mg/day) or vehicle (corn oil) was given by gavage for 4 days. Lipids were assessed in pooled gallbladder biles (n = 5 or 6 in each group).

[a] These values are significantly different from those of the control group at $P < 0.05$.

diates the basolateral cholesterol/phospholipid efflux, requiring the presence of apoA-I as stimulator and acceptor of lipid transport to the basolateral compartment (57). The heterodimeric complex ABCG5/G8, which has a role in canalicular secretion of cholesterol from the hepatocyte (62, 63) and cholesterol efflux in the small intestine

Fig. 9. Megalin expression was upregulated by a lithogenic diet, but not by cholesterol. Mice (five per group) were fed for 10 days with a control diet, a lithogenic diet, a 2% cholesterol-enriched diet, or a 0.5% (w/w) CA-enriched diet. A: Megalin immunoblot analysis of GST-RAP pull downs of gallbladder extracts (450 μg of protein from pooled gallbladders from each group). B: To evaluate cubilin expression, 100 μg aliquots of each gallbladder extract were immunoblotted with cubilin antibody. C: As a control for the effects of the diets, the expression level of scavenger receptor class B type I (SR-BI) was determined from 100 μg of each gallbladder extract.

(64), is also present in human gallbladder, located intracellularly and apically in GBECs (61). This ABC complex probably acts as a molecular sensor of gallbladder intracellular cholesterol content, participating in the apical efflux of cholesterol into bile in a way that counteracts the apical cholesterol absorption process. This latter process may involve megalin/cubilin-mediating lipid absorption/transport processes via the uptake of lipoproteins such as apoA-I. ApoA-I is secreted by GBECs (52, 58) as well as apoE, another lipid transport protein that is a megalin ligand. ApoJ has a high affinity for megalin (53) that is increased after the association with lipids, and we find it expressed and secreted by GBECs, consistent with the in situ hybridization data of others (65). In addition to targeting apolipoprotein-cholesterol complexes to lysosomes for hydrolysis, megalin might also mediate cholesterol trafficking to the basolateral domain of GBECs for secretion, avoiding an accumulation of large amounts of intracellular cholesterol that could damage GBE itself and/or adjacent muscle cells and thus reduce gallbladder motility. In fact, it has been shown that megalin mediates the transcytosis of some ligands in kidney and thyroid epithelial cells (66, 67). However, an orchestrated participation of other lipid transporters expressed in GBECs, such as ABCA1, has to be considered (57).

Calcium is an element extremely important in the pathogenesis of gallstones. Calcium salts formed with the calcium-sensitive ions bilirubinate, carbonate, and phosphate, which are major components of pigment gallstones and are present at high concentrations in the centers and rims of cholesterol gallstones (68, 69). To know the factors that regulate calcium solubility in bile is thus essential to understand the process that leads to gallstone formation. In this regard, besides the potential role of megalin in lipid absorption from the bile, a role in biliary calcium salt levels could be ascribed to this receptor. Megalin plays an important role in calcium metabolism, acting as a receptor that directly binds calcium (70), and also as a regulator of systemic calcium homeostasis, as a result of its role in the internalization of 25-(OH) vitamin D3 in complex with vitamin D binding protein (71). The most abundant biliary calcium salt is calcium carbonate, which is precipi-

tated when the bicarbonate concentration increases because of inadequate acidification of the bile (72). Thus, the correct acidification of alkaline hepatic bile in the gallbladder is a crucial event to prevent calcium precipitation and gallstone formation. Carbonic anhydrases (73) and NHE3 (7–9) are gallbladder proteins involved in the bile acidification mechanisms. In kidney proximal tubules, the activity of NHE3 is negatively regulated by megalin (27). It is therefore reasonable to speculate that megalin, in GBE, could also regulate NHE3 activity and eventually the activity of carbonic anhydrases by modulating its trafficking and compartmentalization, thus influencing the acidification of alkaline hepatic bile.

Genetic data in mice suggest a role for megalin in gallstone disease, because the megalin gene is within the *Lith1* locus (34). The availability of megalin-deficient mice might help to address this question. Although most megalin-deficient mice die, those that survive could perhaps be used to test the role of this megalin in gallstone formation (74). The development of crystals of cholesterol in bile would require that 2 month old animals be fed with a lithogenic diet for at least 2 weeks. This treatment may or may not be tolerated by the fragile knockout animals.

In the present study, we evaluated the regulation of megalin expression under conditions that induce cholesterol gallstone formation or a change in the composition of the BA pool. Because it is known that megalin expression is positively regulated by vitamin D through VDR (55) and recently it was demonstrated that LCA is a ligand for VDR (41), we decided to explore the possibility that megalin expression was regulated, in vitro and in vivo, by BAs as LCA. In vitro, megalin expression was greatly decreased at LCA concentrations that activate its known mediator FXR (>25 µM) and was slightly increased or not changed by lower concentrations of LCA that activate VDR (5–10 µM) (41). In vivo, the expression of megalin but not cubilin was decreased by ~40% by LCA and was increased severalfold by the natural FXR agonists CA and CDCA. At the same time, we also found the FXR was strongly expressed in gallbladder and that its expression was negatively regulated by LCA. Noteworthy, the regulation of megalin expression by FXR ligands was similar to that described for BSEP, with LCA acting as an antagonist (75), and guggulipid and its active component guggulsterone (56), which normally have an FXR antagonist effect (76), acting with a selective FXR agonist effect. Analyzing the promoter gene sequence available for megalin (77), we found two putative FXR/RXR binding sites, suggesting that the megalin gene could be a target for this nuclear receptor, as are other genes related to lipoprotein metabolism (78, 79). FXR itself was recently shown to be genetically associated with cholesterol gallstone formation in mice (80).

The composition of the BA pool is abnormal in lithiasic patients; LCA concentration is increased and CDCA is decreased compared with normal (81). Megalin expression in gallbladder could thus be downregulated by lithogenic bile, enriched in LCA and poor in CDCA, favoring a condition of lower cholesterol gallbladder absorption, among other events. Cholesterol gallstone dissolution therapies

have included patient treatment with ursodeoxycholic acid alone (82) or in combination with the FXR agonist CDCA (83, 84), based mostly on the physicochemical properties of these BAs. Many reports indicate that CDCA is more efficient in gallstone dissolution and in decreasing the CSI and stone calcification (83, 84). Considering that ursodeoxycholic acid does not activate FXR (41), it is tempting to speculate that part of the therapeutic effect of the CDCA could be explained by its enhanced megalin expression in the GBE. This in turn could lead to subsequent improvement in the cholesterol absorption process, calcium remotion, and/or other mechanism(s) that could inhibit and control gallstone formation.

We also assessed the effect of feeding the animals a lithogenic diet on the gallbladder expression of megalin and cubilin. Megalin expression was significantly increased by the lithogenic diet as well as by a diet containing 0.5% CA, compared with the controls. This suggests that a compensatory mechanism operates under conditions of high cholesterol concentration in gallbladder bile. Biliary cholesterol levels and the CSI were similarly increased after CA- or cholesterol-rich diets, but megalin protein expression was increased only by CA, indicating that the expression of megalin was not sensitive to cholesterol levels in bile. This finding is consistent with other reports showing that megalin expression in cultured endodermal cells is not responsive to cholesterol (32). The diet-induced modifications observed for megalin in vivo were opposite to those found for SR-BI, the other apoA-I receptor present at the apical membrane of GBE (40). Megalin and SR-BI could function differently in the context of gallbladder physiology. Indeed, evidence indicates that the absence of SR-BI in mice has no relevance to the propensity to develop gallstones (40).

BA treatments and lithogenic diet dramatically influenced megalin expression but not cubilin expression. Because cubilin trafficking (32) and endocytic function are dependent on the action of megalin, it is not necessary that both proteins be equally regulated. This differential expression regulation has been shown in other systems (85). Indeed, recent findings have shown that in the embryonic yolk sac, megalin and cubilin expression is not strictly linked (23). Addressing the question of the role of cubilin for gallbladder function would probably require the development of cubilin-deficient animals and the study of gallstone development under lithogenic diets.

Until now, besides several long-term investigations, the only gallbladder proteins/genes that have been implicated in downstream defects related to cholesterol gallstone development have been mucin genes (13). It has also been shown that mucin is upregulated by hydrophobic BAs, and its expression level in the gallbladder affects susceptibility to cholesterol gallstone formation in mice. Now, our data allow us to speculate that megalin, like mucin, could have a role in the gallbladder generating susceptibility to cholesterol gallstone formation under specific conditions. All together, our findings strongly suggest that a central role could be played by megalin in the pathophysiology of the gallbladder, making it a potential target for the diag-

nosis and treatment of gallstone disease, a very important matter considering the high prevalence of this disease in Western countries. ⌷

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