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The phospholipid flippase ATP8B1 mediates apical localization of the cystic fibrosis transmembrane regulator

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ABSTRACT

Progressive familial intrahepatic cholestasis type 1 (PFIC1) is caused by mutations in the gene encoding the phospholipid flippase ATP8B1. Apart from severe cholestatic liver disease, many PFIC1 patients develop extrahepatic symptoms characteristic of cystic fibrosis (CF), such as pulmonary infection, sweat gland dysfunction and failure to thrive. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel essential for epithelial fluid transport. Previously it was shown that *CFTR* transcript levels were strongly reduced in livers of PFIC1 patients. Here we have investigated the hypothesis that ATP8B1 is important for proper CFTR expression and function.

We analyzed CFTR expression in ATP8B1-depleted intestinal and pulmonary epithelial cell lines and assessed CFTR function by measuring short-circuit currents across transwell-grown ATP8B1-depleted intestinal T84 cells and by a genetically-encoded fluorescent chloride sensor. In addition, we studied CFTR surface expression upon induction of *CFTR* transcription.

We show that CFTR protein levels are strongly reduced in the apical membrane of human ATP8B1-depleted intestinal and pulmonary epithelial cell lines, a phenotype that coincided with reduced CFTR activity. Apical membrane insertion upon induction of ectopically-expressed CFTR was strongly impaired in ATP8B1-depleted cells.

We conclude that ATP8B1 is essential for correct apical localization of CFTR in human intestinal and pulmonary epithelial cells, and that impaired CFTR localization underlies some of the extrahepatic phenotypes observed in ATP8B1 deficiency.

1. Introduction

ATP8B1 is a class 4 P-type ATPase (P4-ATPase) and a member of a conserved family of proteins that catalyze the translocation of (amino)phospholipids from the exoplasmic to the cytoplasmic leaflet of biological membranes [1]. P4-ATPases are essential for maintaining the asymmetric distribution of phospholipids within biological membranes but also for the biogenesis of transport vesicles in the biosynthetic and endocytic pathways (reviewed in [2–4]).

Mutations in the gene encoding ATP8B1 cause progressive familial intrahepatic cholestasis type 1 (PFIC1), a rare autosomal recessively inherited liver disease [5]. PFIC1 patients usually present at a young age with typical cholestatic symptoms, including elevated serum bile salt, bilirubin and transaminase levels, low gamma-glutamyltranspeptidase levels, and intractable pruritus [6]. Previous in vivo and in vitro studies indicate an important function for ATP8B1 in the maintenance of apical membrane structure of hepatocytes [7,8]. Besides cholestatic liver disease, many PFIC1 patients develop extrahepatic symptoms such as diarrhea, pulmonary infection, defects in sweat gland function and failure to thrive [9–12]. Apart from diarrhea, these symptoms are commonly associated with cystic fibrosis (CF) [13]. CF is the most prevalent genetically inherited disease in Caucasians and is mainly manifested by airway disease [14]. CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride and bicarbonate channel that is expressed in the apical membrane of many epithelial cells, where it plays a principal role in transepithelial water and salt transport [15,16]. CFTR activity is tightly regulated by a multitude of signaling mechanisms and by the balance between endocytic retrieval and

Abbreviations: PFIC1, progressive familial intrahepatic cholestasis type 1; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; Isc, short circuit current; TGN, *trans*-Golgi network; ASBT, apical sodium-dependent bile acid transporter; AP, alkaline phosphatase; SI, sucrose isomaltase; APN, aminopeptidase N.

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insertion from an intracellular vesicular pool (reviewed in [17,18]). Interestingly, ATP8B1 deficiency has been associated with reduced expression of *CFTR*, both in liver of two PFIC1 patients and in an ATP8B1-depleted human biliary epithelial cell line [19].

The observation that PFIC1 patients share similar symptoms of CF patients and that *CFTR* expression is reduced in PFIC1 patients led us to investigate the hypothesis that ATP8B1 deficiency leads to reduced CFTR activity. In this study, we have analyzed the effects of depletion of ATP8B1 in intestinal and pulmonary epithelial cells on the expression and activity of CFTR. Our results underline the importance of ATP8B1 in proper apical surface expression of CFTR in epithelial cells.

2. Materials and methods

2.1. Cell culture and lentiviral transduction

The human colonic carcinoma cell line T84 was cultured in 1:1 DMEM:HAM's F12 (Lonza) supplemented with 16 mM HEPES, pH 7.5 (Sigma-Aldrich), 14 mM $NaHCO_3$ (Sigma-Aldrich), 10% bovine calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin at 37 °C in a 10% CO_2 humidified atmosphere. The human colorectal adenocarcinoma cell line Caco-2 and the lung adenocarcinoma cell line Calu-3 were cultured in respectively DMEM (Lonza) and Alpha MEM (Lonza) supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Stable ATP8B1 knockdown ('ATP8B1.7') and control ('control') cell lines were generated by lentiviral transduction with validated MISSION shRNA (Sigma-Aldrich) vectors as described previously [20]. TRCN0000050127 was used to target *ATP8B1* and a scrambled, non-targeting shRNA SHC002 was used to generate control cells. Cells were incubated with virus-containing supernatants/DMEM (1:1) supplemented with 10 μg/ml diethylaminoethyl-dextran for 4 h after which the medium was refreshed. Two days post-transduction, cells were selected on 10 μg/ml (Caco-2, T84) or 2 μg/ml (Calu-3) puromycin. Knockdown of ATP8B1 was checked regularly in subsequent passages via immunoblotting and quantitative RT-PCR. Cell lines overexpressing CFTR (driven by a cytomegalovirus (CMV) promoter) [21] or a ratiometric chloride sensor (driven by a CMV promoter [22]) were generated by lentiviral transduction. All experiments were performed on cells grown on microporous polycarbonate membrane filters (12 mm diameter transwell, 0.4 μm pore size, Corning) or on cells grown as a monolayer at 3 weeks post-confluency.

2.2. Animals

Male, age-matched (3–6 months) $Atp8b1^{G308V/G308V}$ mutant and wild type mice in a C57BL/6 background [23] were kept in a pathogen-free environment on a controlled 12-h light/dark regimen in the animal facility of the Academic Medical Center (AMC). All animal experiments were approved by the institutional animal care and use committee of the AMC.

2.3. Ussing chamber experiments

Mouse intestinal explants were isolated as follows: mice were anesthetized. The intestines from jejunum to colon were removed and flushed with ice cold PBS. The mucosal layer was turned inside out and the muscular layer was stripped with a cotton swab. Intestinal segments and cut-open gallbladders were mounted in an Ussing chamber setup (Physiological Instruments Inc). For transwell-grown T84 monolayers, filters were excised from their holders and mounted in an Ussing chamber setup. Ussing chamber experiments were performed as follows: samples were incubated in modified Meyler buffer (128 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.33 mM Na_2HPO_4, 10 mM HEPES). Serosal/basolateral buffer for the assessment of apical CFTR activity contained equimolar amounts of Na^+-gluconate and K^+-gluconate instead of NaCl and KCl. Buffers and samples in the Ussing chamber were incubated at 37 °C and continuously gassed with carbogen. Voltage over the explant or transwell was clamped at 0 during experiments. Electrical currents were measured on a DVC-1000 (World Precision Instruments), digitalized with Powerlab 4/26 (AD Instruments) and analyzed with Labchart 6 (AD Instruments).

2.4. Ratiometric intracellular chloride measurements

T84 cells stably expressing a ratiometric chloride sensor [22] were grown on glass coverslips. After 2 weeks of confluence the glass slides were mounted in a perfusion chamber at 37 °C. Cells were perfused with equilibration buffer (144 mM NaCl, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM Ca gluconate, 5 mM HEPES pH 7.5, 5.6 mM D-glucose) for 10 min before the start of an experiment. NaI buffer contained equimolar amounts of NaI instead of NaCl. Buffers were changed after a stable fluorescence emission was reached (typically after 10–15 min). Fluorescence was detected on a NOVOSTAR microplate reader (BMG Labtech) with excitation filters of 440 ± 10 nm and 485 nm and an emission filter of 540 ± 10 nm. Fluorescence was acquired every 5 s. After background subtraction, fluorescence emission of the YFP excited signal at 485 nm was divided by fluorescence emission of the CFP excited signal at 440 nm. Ratio changes over time were calculated by subtracting the averages of 3 sequential measurement points at stabilized fluorescence emissions before and after buffer change or addition of forskolin.

2.5. Quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). cDNA was synthesized from 2 μg of total RNA with oligo dT 12–18 and random hexamer primers and Superscript III RT (Invitrogen). RT-PCR measurements were performed on a Lightcycler 480 (Roche) with SYBR-Green master mix (Roche). Expression levels of *ATP8B1* or *CFTR* were calculated with the LinregPCR program [24] using human acidic ribosomal phosphoprotein P0 (*RPLP0*) or mouse villin (*Vil1*) as a reference gene. Primer sequences used: *ATP8B1*: forward 5′-GTCTTGGACAGAGTCACT TC-3′, reverse 5′- CGTCTTATCAGAGAAGATATAAT-3′; human *CFTR*: forward 5′-ACCTGGGAAAGGGCTGTTAT-3′, reverse 5′-CCTGGAGCTTCT GTGGAAAG-3′; human *RPLP0*: forward 5′-TCGACAATGGCAGCATCTAC-3′, reverse 5′-ATCCGTCTCCACAGACAAGG-3′; mouse *Cftr*: forward 5′-GCACAGCAGCTCAAACAACTGGAA, reverse 5′-TTCTCATTTGGAACCAGC GCAA-3′; mouse *Vil1*: forward 5′-GCTCCAACCAGACCGGACGC-3′, reverse 5′-GGGGCTCGTGTCCCTGCTTC-3′.

2.6. Cell surface biotinylation

Cells were washed 3× with ice-cold phosphate buffered saline (pH 8.2) containing 1 mM $MgCl_2$/0.1 mM $CaCl_2$ (PBS-CM) and were incubated with 1 mg/ml sulfo-NH-ss biotin (Thermo Scientific) in ice-cold PBS-CM for 60 min while gently shaken. To quench the non-bound biotin, cells were washed twice for 5 min with ice-cold PBS-CM containing 100 mM glycine and once more with ice-cold PBS-CM. Cells were lysed for 30 min on ice in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche). Biotinylated protein was precipitated with high-capacity neutravidin agarose resin (Thermo Scientific) for 2 h at 4 °C. Beads were washed four times with RIPA buffer and in between washes spun down for 1 min at 450×*g*. Biotinylated protein was eluted with DTT- and SDS-containing sample buffer at room temperature. Total lysates and eluates were analyzed by SDS-PAGE and western blotting.