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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Decreased renal accumulation of aminoglycoside reflects defective receptor-mediated endocytosis in cystic fibrosis and Dent's disease

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Abstract The clinical use of aminoglycoside (AG) antibiotics is limited by their renal toxicity, which is caused by drug accumulation in proximal tubule (PT) cells. Clinical studies reported that renal clearance of AG is enhanced in cystic fibrosis (CF) patients, which might reflect the role of CFTR in PT cell endocytosis. In order to assess the role of chloride transporters on the renal handling of AG, we investigated gentamicin uptake and renal accumulation in mice lacking functional CFTR (*Cftr*$^{\Delta F/\Delta F}$) or knock-out for the Cl^-/H^+ exchanger ClC-5 (*Clcn5*$^{Y/-}$). The latter represent a paradigm of PT dysfunction and defective receptor-mediated endocytosis. As compared with controls, *Cftr*$^{\Delta F/\Delta F}$ and *Clcn5*$^{Y/-}$ mice showed a 15% to 85% decrease in gentamicin accumulation in the kidney, respectively, in absence of renal failure. Studies on primary cultures of *Cftr*$^{\Delta F/\Delta F}$ and *Clcn5*$^{Y/-}$ mouse PT cells confirmed the reduction in gentamicin uptake, although colocalization with endosomes and lysosomes was maintained. Quantification of endocytosis in PT cells revealed that gentamicin, similar to albumin, preferentially binds to megalin. The functional loss of ClC-5 or CFTR was reflected by a decrease of the endocytic uptake of gentamicin, with a more pronounced effect in cells lacking ClC-5. These results support the concept that CFTR, as well as ClC-5, plays a relevant role in PT cell endocytosis. They also demonstrate that the functional loss of these two chloride transporters is associated with impaired uptake of AG in PT cells, reflected by a decreased renal accumulation of the drug.

Keywords Chloride channel · Endocytosis · Kidney · Proximal tubule · Epithelial transport

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Introduction

Aminoglycosides (AG) are among the most commonly used antibiotics worldwide. Their activity against a wide range of Gram-negative bacteria combined with a low rate of reversible resistance makes AG the drug of choice to treat life-threatening infections. However, nephrotoxicity of AG, which is observed in up to 25% of treated patients, represents a serious adverse event and is a dose-limiting factor for their therapeutic use [1, 18]. AG are commonly used to treat exacerbations of pulmonary infections (particularly with *Pseudomonas aeruginosa*) in patients with cystic fibrosis (CF). Nephrotoxicity of AG is particularly burdensome in such patients, considering the tenacity of infections and the necessity for repeated treatment courses [25]. A further aspect is that CF patients show enhanced renal

clearance for many drugs including AG [13, 30]. The molecular basis of such enhanced drug clearance in CF—increase in filtration, decrease in tubular reabsorption or increase in tubular secretion—is debated and is probably drug-specific [24].

Aminoglycosides are non-protein-bound drugs characterized by low tissue penetrance and free glomerular filtration. Around 80% of the administered dose is excreted into the urine within 24 h. However, up to 5% of the dose is reabsorbed in the proximal tubule (PT), where the drug accumulates for a long time (half-life >100 h), leading to renal damage [23, 33]. Aminoglycoside-induced kidney failure is typically non-oliguric, with evidence of PT dysfunction (renal Fanconi syndrome) and occasional toxicity in more distal tubular segments, as reflected by NaCl or magnesium wasting. However, accumulation within the renal cortex, especially in PT cells, is thought to play a key role in the pathogenesis of AG nephrotoxicity [14].

Experiments in rat models have revealed that accumulation of AG in PT cells is mediated by the multi-ligand receptor, megalin [17]. Megalin and its co-receptor cubilin are abundantly expressed at the apical surface of PT cells, where they mediate the main endocytic pathway for clearance of low-molecular-weight (LMW) proteins that are ultrafiltered by the glomerulus [4]. Rare human disorders caused by loss-of-function mutations in the genes coding for megalin or cubilin, as well as animal models with invalidated megalin or cubilin receptors, are invariably associated with urinary loss of LMW ligands [6]. Apart from these receptors, recent studies have revealed that intravesicular chloride transporters may also play an important role in the reabsorption of ultrafiltered ligands [12]. In particular, the vesicular Cl^-/H^+ exchanger, ClC-5, is crucial for receptor-mediated endocytosis and megalin/cubilin trafficking in PT cells [5, 20, 32]. Mutations of the *CLCN5* gene that encodes ClC-5 cause Dent's disease (OMIM #30009), a rare X-linked renal tubulopathy characterized by a generalized dysfunction of the PT associated with LMW proteinuria [8]. More recently, Jouret et al. have demonstrated that loss-of-function mutations in the CF transmembrane conductance regulator (*CFTR*, *ABCC7*) gene induce a moderate but significant PT alteration of LMW protein handling [11]. Indeed, in mouse kidney, CFTR co-distributes with ClC-5 and the vacuolar-ATPase in endosomes located in the apical area of PT cells. Furthermore, lack of CFTR in renal PT cells induces instability of cubilin at the brush border, leading to its accelerated shedding into urine and LMW proteinuria in CF mice and patients [11]. Accordingly, a reduced uptake of AG by PT cells could provide a mechanistic explanation for the enhanced renal clearance of AG observed in CF.

In order to test this hypothesis and investigate kidney handling of AG, we performed in vivo and in vitro studies to characterize gentamicin handling by PT cells. We used two clinically relevant animal models, i.e. mice lacking functional CFTR ($Cftr^{\Delta F/\Delta F}$) and those knock-out for ClC-5 ($Clcn5^{Y/-}$), a well-established model for Dent's disease and defective PT cell endocytosis.

Materials and methods

Mouse models

Experiments were conducted on age-matched and gender-matched $Cftr^{+/+}$ and mutated $Cftr^{\Delta F/\Delta F}$ mice (FVB/129 background) [31], and $Clcn5^{Y/+}$ and $Clcn5^{Y/-}$ mice (C57BL6 background) [32]. All mice had free access to appropriate standard diet (Carfil Quality, Oud-Turnhout, Belgium). The tap water of the *Cftr* mice was enriched with Movicol (55.2 g/L; Norgine, Heverlee, Belgium). The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Université catholique de Louvain.

Renal function and gentamicin handling

Mice were injected with a therapeutic dose (5 mg/kg body weight) of gentamicin (Schering-Plough, Brussels, Belgium) [9]. Blood was obtained by retro-orbital puncture after 30 min, under anesthesia with Anesketin (Eurovet, Brussels, Belgium) and Rompun (Bayer, Brussels, Belgium). Subsequently mice were kept in metabolic cages for 24 h with ad libitum access to food and drinking water. Urine was collected, and blood was obtained by puncture of the vena cava at the time of killing. Plasma and urine concentrations of gentamicin were determined using an AxSYM System (detection limit: 0.2 μg/mL; Abbott Laboratories, Abbott Park, IL, USA). Plasma gentamicin concentration at the time of sacrifice was undetectable, and therefore only the peak concentration at 30-min post-injection was assessed. Biological parameters in plasma and urine were measured with a Synchron CX5 analyzer (Beckman Coulter, Fullerton, CA), whereas Clara Cell protein (CC16) concentration, a 16-kD marker of PT dysfunction, was determined in duplicate by latex immunoassay [32].

A kidney was split in two parts; half was fixed for 6 h at 4° C in 4% 0.1 M phosphate buffered (pH 7.4) formaldehyde (Boehringer Ingelheim, Heidelberg, Germany), and the other half was flash-frozen and stored at −80°C. The contralateral kidney was homogenized in 0.25 M sucrose and 3 mM imidazole buffer (pH 7.4) that contained Complete (Roche), in

a Potter-Elvehjem tissue homogenizer (Thomas Scientific, Swedsboro, NJ) [11]. The homogenate was centrifuged at 1000× *g* for 15 min at 4°C, and the supernatant was analyzed biochemically for gentamicin concentration using the AxSYM System. To confirm the early accumulation of gentamicin in PT cells, some mice were sacrificed 10 min after injection of gentamicin (5 mg/kg body weight), with fixation of kidney samples as described previously.

Primary cell culture

Primary mouse proximal tubule cells (mPTC) were cultured under sterile conditions from collagenase-digested cortical fragments of kidneys isolated from male *Clcn5* and *Cftr* mice as previously described [27, 29]. Seven-day-old confluent monolayers, which retain their differentiation and endocytic capacity [21], were used for experiments.

Immunostaining

Kidney samples and mPTC were fixed in 4% formaldehyde before embedding in paraffin as described [11]. After incubation with 10% serum, kidney sections or mPTC were incubated with primary antibodies diluted in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). After washing, sections were incubated with biotinylated secondary anti-IgG antibodies (Vector Laboratories, Brussels, Belgium). Sections were viewed under a Leica DMR coupled to a Leica DC300 digital camera (Leica, Heerbrug, Switzerland). For immunofluorescence, Alexa-conjugated secondary anti-IgG antibodies were used (Invitrogen, Merelbeke, Belgium). Nuclei were stained with DraQ5™ (Biostatus, UK). Filters cut from the holder or kidney sections were mounted in Prolong Gold Anti-fade reagent (Invitrogen, Belgium). Sections were analyzed under a Zeiss LSM510Meta Confocal microscope (Zeiss, Belgium).

Antibodies

Immunostaining analyses were performed using well-characterized rabbit monoclonal antibody against gentamicin (a gift from K. Fujiwara, Sojo University, Japan) [10], mouse monoclonal antibody against early endosomal antigen-1 (EEA1), and polyclonal antibodies against megalin (a gift from P. Verroust, INSERM, Paris, France).

Gentamicin endocytosis

Characterization of the gentamicin uptake was performed in fixed and live mPTC. Confluent monolayers of mPTC, isolated from *Clcn5* and *Cftr* mice, were exposed to 5 mg/mL gentamicin for 7 or 45 min at 37°C, fixed in 4% formaldehyde (see "Immunostaining") and subsequently stained using anti-EEA1 and anti-gentamicin antibodies. For live-cell imaging, confluent monolayers of mPTC were simultaneously incubated with 1 mg/mL Alexa 488-gentamicin and Lysotracker Red (Invitrogen, Merelbeke, Belgium) for 7 min at 37°C and washed. Fluorescence was chased up to 45 min after exposure of the cells to gentamicin. Cells were examined using the Zeiss LSM510Meta confocal microscope.

Quantification of endocytosis was performed as previously described [21]. Confluent mPTC were incubated with Alexa488-conjugated gentamicin or Alexa488-conjugated BSA (both at 0.5 mg/mL) for 15 min at 37°C. After incubation, mPTC were washed five times with cold HBSS (4°C) and lysed. The fluorescence in the lysate was measured in a fluorimeter (Perkin Elmer) with excitation at 490±10 nm and emission at 525±10 nm. For metabolic inhibition experiments, mPTC were incubated with 10 mM sodium azide or 50 mM 2-deoxyglucose (DOG) for 30 min at 37°C before the endocytic assay. For competition experiments, mPTC were simultaneously incubated with 50 mM unlabelled albumin or transferrin. Fluorescence was normalized for the amount of protein in the cells, measured using the BCA kit (Pierce, Belgium).

Data are expressed as mean±SEM. Significance of differences was assessed by unpaired, two-tailed Student's *t* test.

Results

Renal function and gentamicin handling in *Cftr* and *Clcn5* mice

We first investigated renal function and gentamicin concentrations in $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ mice in comparison with their control littermates. Renal function as measured by creatinine clearance, BUN and plasma creatinine levels was similar for CFTR and ClC-5 mice as compared with wild-type controls (Table 1). The $Cftr^{\Delta F/\Delta F}$ mice showed a significant increase in the urinary albumin excretion as well as LMW proteinuria as evidenced by the increased urinary excretion of CC16. The $Clcn5^{Y/-}$ mice also presented with albuminuria and LMW proteinuria, in a greater extent than that observed in $Cftr^{\Delta F/\Delta F}$ mice. The *Clcn5* knock-out mice also showed polyuria (Table 1).

As for gentamicin handling, the peak plasma concentration of gentamicin was similar in wild-type and $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ mice, indicating that the mice received a similar dose of the drug (Table 2). In contrast, the gentamicin concentration in the kidney 24 h after injection was significantly lower in $Cftr^{\Delta F/\Delta F}$ mice (~15% decrease) and in $Clcn5^{Y/-}$ mice (~85% decrease) (Table 2). These

Table 1 Clinical and biochemical parameters in *Cftr* and *Clcn5* mice

	$Cftr^{+/+}$	$Cftr^{\Delta F/\Delta F}$	$ClCn5^{Y/+}$	$Clcn5^{Y/-}$
Body weight (g)	26.2±1.3	25.2±0.9	24.6±0.4	29.1±0.4[b]
BUN (mg/dL)	25±0.5	27±3.3	27±2.8	25±1.7
Plasma creatinine (mg/dL)	0.072±0.02	0.076±0.02	0.086±0.02	0.08±0.01
Diuresis (μL/24 h)	708±87	704±123	996±176	2317±447[b]
Creatinine clearance (μL/min·g BW)	37±10	40±7	17±4	23±3
Urinary albumin (μg/g creat)	1893±1385	10,869±3513[a]	798±761	58,608±3041[c]
Urinary CC16 (μg/g creat)	42±23	132±20[a]	1.7±0.9	37,665±10,877[d]

There were 5 pairs of age-matched and gender-matched mice per genotype

BUN blood urea nitrogen, *creat* creatinine

[a] $p<0.05$ vs. $Cftr^{+/+}$

[b] $p<0.05$ vs. $Clcn5^{Y/+}$

[c] $p<0.0001$ vs. $Clcn5^{Y/+}$

[d] $p<0.01$ vs. $Clcn5^{Y/+}$

results show that, in absence of renal failure, renal accumulation of gentamicin is significantly decreased in mouse models of CF and Dent's disease in parallel with the degree of PT dysfunction in these models.

Immunolocalization of gentamicin and megalin in *Cftr* and *Clcn5* kidneys

To substantiate the handling data, we performed immunostaining for gentamicin (Fig. 1a) on kidney sections taken from *Cftr and Clcn5* mice 24 h post-injection. Gentamicin was clearly detectable in PT sections of both $Cftr^{+/+}$ and $Clcn5^{Y/+}$ kidneys, contrasting with decreased immunoreactivity in $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ kidneys. A residual, faint signal for gentamicin could be observed in PT profiles from the $Cftr^{\Delta F/\Delta F}$ while no residual signal was detected in $Clcn5^{Y/-}$ mice. Since gentamicin preferentially binds to megalin, we investigated the immunoreactivity for megalin in these very kidney sections from *Cftr* and *Clcn5* mice (Fig. 1b). In comparison with wild-type kidneys, only a faint signal for megalin could be observed at the apical region of PT in $Clcn5^{Y/-}$ kidneys, whereas megalin expression was slightly reduced in $Cftr^{\Delta F/\Delta F}$ kidneys. Immunofluorescence performed shortly (10 min) after injection confirmed the gentamicin uptake in the apical region of PT from $Cftr^{+/+}$ kidneys, with an attenuated immunoreactivity in $Cftr^{\Delta F/\Delta F}$ PT profiles (Fig. 1c). These in vivo data suggest that the decreased renal accumulation of gentamicin could result from a decreased receptor-mediated uptake in PT cells.

Table 2 Gentamicin handling by *Cftr* and *Clcn5* mice

	$Cftr^{+/+}$	$Cftr^{\Delta F/\Delta F}$	$ClCn5^{Y/+}$	$Clcn5^{Y/-}$
Plasma gentamicin (μg/mL)	7.2±0.5	7.9±1.2	6.7±0.6	8.8±0.7
Kidney gentamicin (μg/mL)	0.89±0.1	0.75±0.1[a]	0.66±0.1	0.1±0.05[b]

Plasma gentamicin is peak values obtained at 30 min post-injection; kidney gentamicin is measured at 24 h post-injection. There were 5 pairs of age-matched and gender-matched mice per genotype

[a] $p<0.05$ vs. $Cftr^{+/+}$

[b] $p<0.0001$ vs. $Clcn5^{Y/+}$

Uptake of gentamicin in mPTC from *Clcn5* and *Cftr* mice

We used a well-characterized mPTC culture system [29] to evaluate the subcellular handling of gentamicin and its dependence on CFTR and ClC-5 in PT cells. Primary cultures of mPTC obtained from wild-type (Fig. 2a–d), $Clcn5^{Y/-}$ (Fig. 2e–h), and $Cftr^{\Delta F/\Delta F}$ (Fig. 2i–l) mice were exposed to gentamicin at 37°C for 7 min (early endocytosis) and 45 min (late endocytosis and lysosomal processing). In comparison with wild-type mPTC (Fig. 2a, b), the gentamicin uptake after 7 min was strongly reduced in $Clcn5^{Y/-}$ cells (Fig. 2e, f), while a milder reduction was observed in $Cftr^{\Delta F/\Delta F}$ mPTC (Fig. 2i, j). At 45 min, numerous large gentamicin-positive vesicles could be observed in wild-type mPTC (Fig. 2c, d). A smaller number of positive vesicles were detected in $Clcn5^{Y/-}$ (Fig. 2g, h) and $Cftr^{\Delta F/\Delta F}$ (Fig. 2k, l) mPTC.

We next used mPTC to identify the localization of gentamicin within the endosomal–lysosomal pathway. Monolayers of mPTC were double stained for gentamicin and anti-early endosomal antigen-1 (EEA1) after incubation with gentamicin for 7 min (Fig. 3). In wild-type mPTC, gentamicin colocalized with EEA1, confirming its presence in the early endosomes (Fig. 3a–c). By contrast, almost no gentamicin could be detected in $Clcn5^{Y/-}$ mPTC (Fig. 3d), whereas a markedly reduced staining was detected in

$Cftr^{\Delta F/\Delta F}$ mPTC (Fig. 3g). The few vesicles that contained gentamicin stained positive for EEA1, indicating the involvement of early endosomes (Fig. 3i).

A pulse-chase experiment was performed to investigate the progression of gentamicin into the lysosomal compartment of mPTC (Fig. 4). Monolayers of wild-type (Fig. 4a–c), $Clcn5^{Y/-}$ (Fig. 4d–f), and $Cftr^{\Delta F/\Delta F}$ mPTC (Fig. 4g–i) were exposed to Alexa 488-gentamicin and Lyso Tracker Red, and gentamicin localization was analyzed after 45 min. In wild-type mPTC, gentamicin colocalized with LysoTracker Red (Fig. 4c) confirming intra-lysosomal capture. A reduction of gentamicin uptake was detected in $Clcn5^{Y/-}$ and $Cftr^{\Delta F/\Delta F}$ mPTC (Fig. 4d, g, respectively), with occasional colocalization of few gentamicin-positive

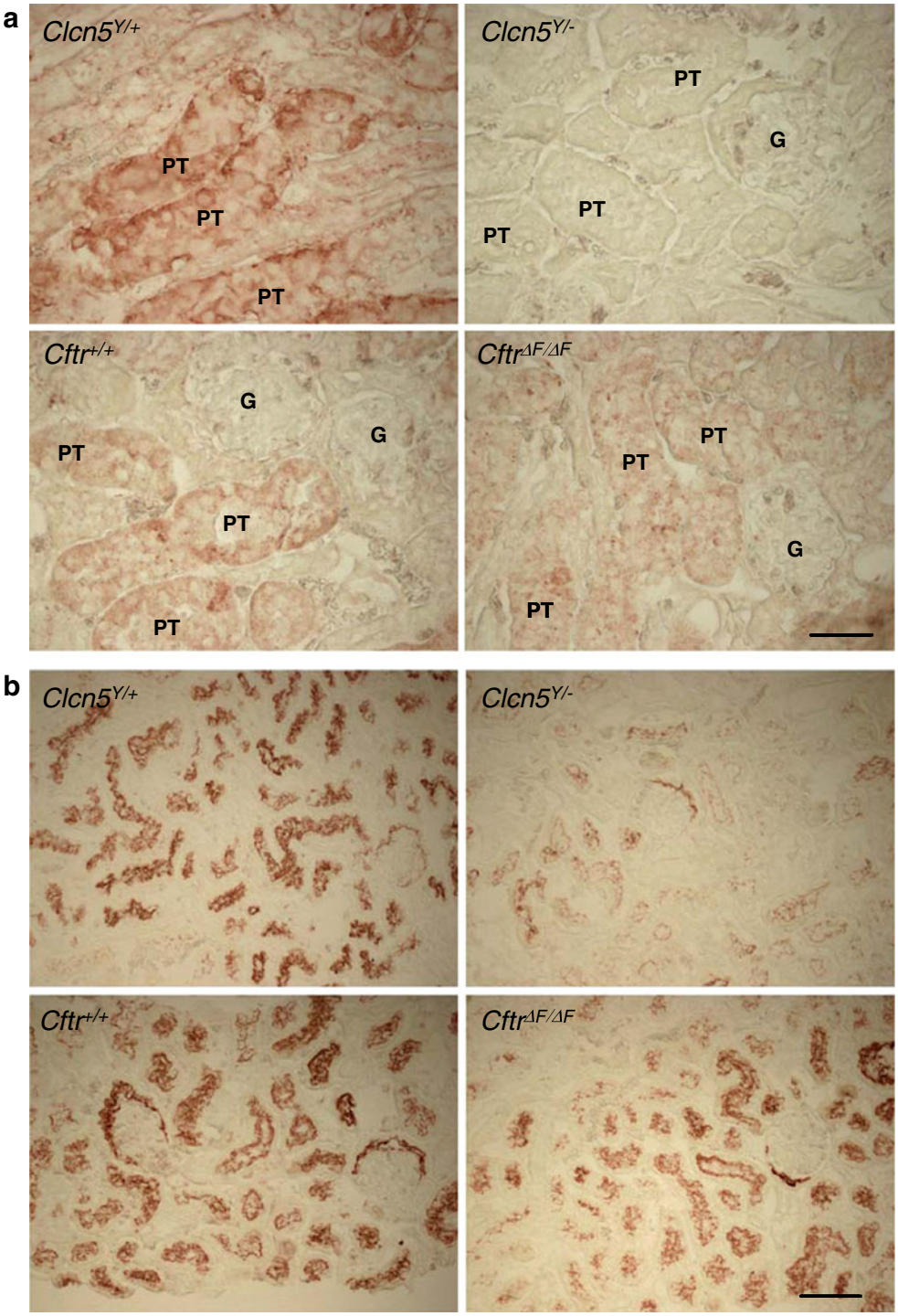

Fig. 1 Gentamicin uptake and megalin expression in *Cftr* and *Clcn5* kidneys. **a** Immunostaining for gentamicin in *Cftr* and *Clcn5* kidneys 24 h post-injection of gentamicin (5 mg/kg) showing segmental gentamicin staining in the proximal tubules (*PT*) of both wild-type $Cftr^{+/+}$ and $Clcn5^{Y/+}$ kidneys. In contrast, the staining is abolished in the proximal tubules of $Clcn5^{Y/-}$ kidneys and is weakly detected in $Cftr^{\Delta F/\Delta F}$ kidneys. **b** Immunostaining for megalin showing a strong expression at the apical region of the cells lining the proximal tubules in the wild-type $Cftr^{+/+}$ and $Clcn5^{Y/+}$ kidneys. In contrast, the staining is almost undetectable in $Clcn5^{Y/-}$ kidneys and is slightly decreased in $Cftr^{\Delta F/\Delta F}$ kidneys. Scale bar: 50 μm (**a**), 100 μm (**b**). **c** Immunofluorescent staining for gentamicin in *Cftr* kidneys 10 min post-injection. Positive signal for gentamicin is observed in the subapical area of the cells lining proximal tubules (PT) of $Cftr^{+/+}$ kidneys. The signal is attenuated in the proximal tubules of $Cftr^{\Delta F/\Delta F}$ kidneys. Scale bar: 50 μm (**a**), 100 μm (**b** and **c**)

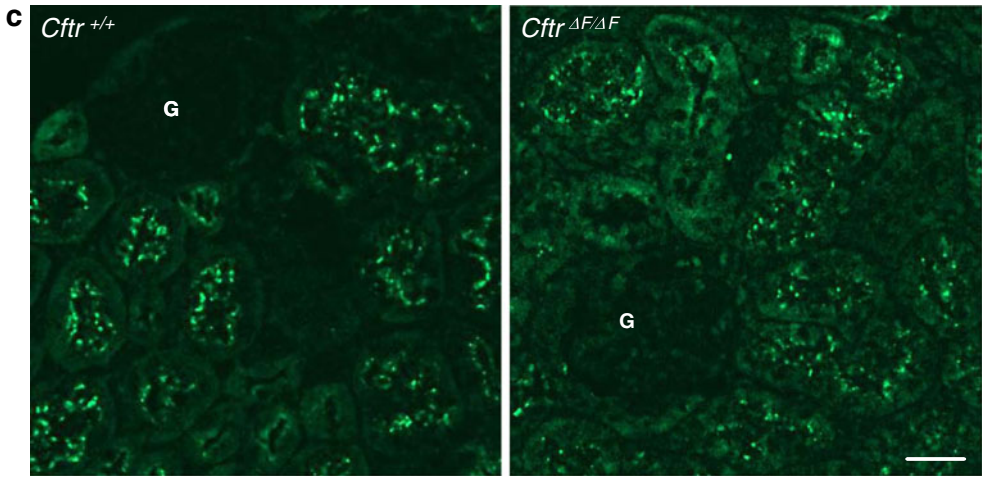

Fig. 1 continued.

vesicles with the lysosomal marker (Fig. 4f, i). Taken together, these data demonstrate that uptake of gentamicin and its progression along the endosomal–lysosomal pathway are affected by the functional loss of CFTR or ClC-5 in PT cells.

Quantification of gentamicin endocytosis in mPTC from *Clcn5* and *Cftr* mice

To monitor the effect of CFTR or ClC-5 loss on gentamicin uptake, we incubated mPTC from *ClCn5* and *Cftr* mice for

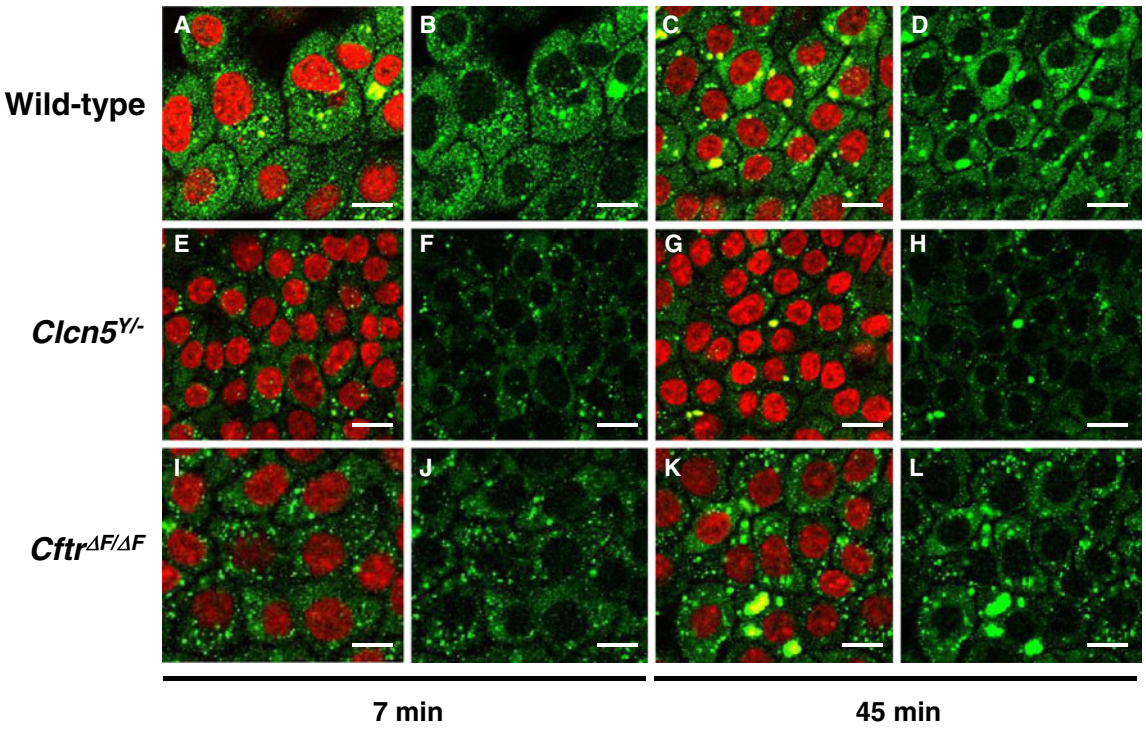

Fig. 2 Gentamicin uptake in proximal tubule cells. Primary cultures of proximal tubule cells (mPTC) obtained from $Clcn5^{Y/+}$ (wild-type, **a**–**d**), $Clcn5^{Y/-}$ (**e**–**h**), and $Cftr^{\Delta F/\Delta F}$ (**i**–**l**) kidneys exposed to 5 mg/mL gentamicin (37°C for 7 min or 45 min). Staining with anti-gentamicin and labeling with Alexa488 IgG were analyzed by confocal microscopy. Nuclei counterstained with DraQ5™ (*red*). In comparison with wild-type mPTC (**a**–**b**), $Clcn5^{Y/-}$ cells showed a drastically reduced early (7 min) uptake of gentamicin (**e**–**f**), while a milder reduction was observed in $Cftr^{\Delta F/\Delta F}$ mPTC (**i**–**j**). At 45 min, numerous large gentamicin-positive vesicles could be observed in wild-type mPTC (**c**–**d**). A smaller number of positive vesicles were detected in $Clcn5^{Y/-}$ (**g**–**h**) and $Cftr^{\Delta F/\Delta F}$ (**k**–**l**) mPTC. No difference in staining could be observed between mPTC obtained from wild-type $Clcn5^{Y/+}$ and $Cftr^{+/+}$ kidneys, and therefore a representative image of wild-type mPTC is shown. Scale bar: 10 μm

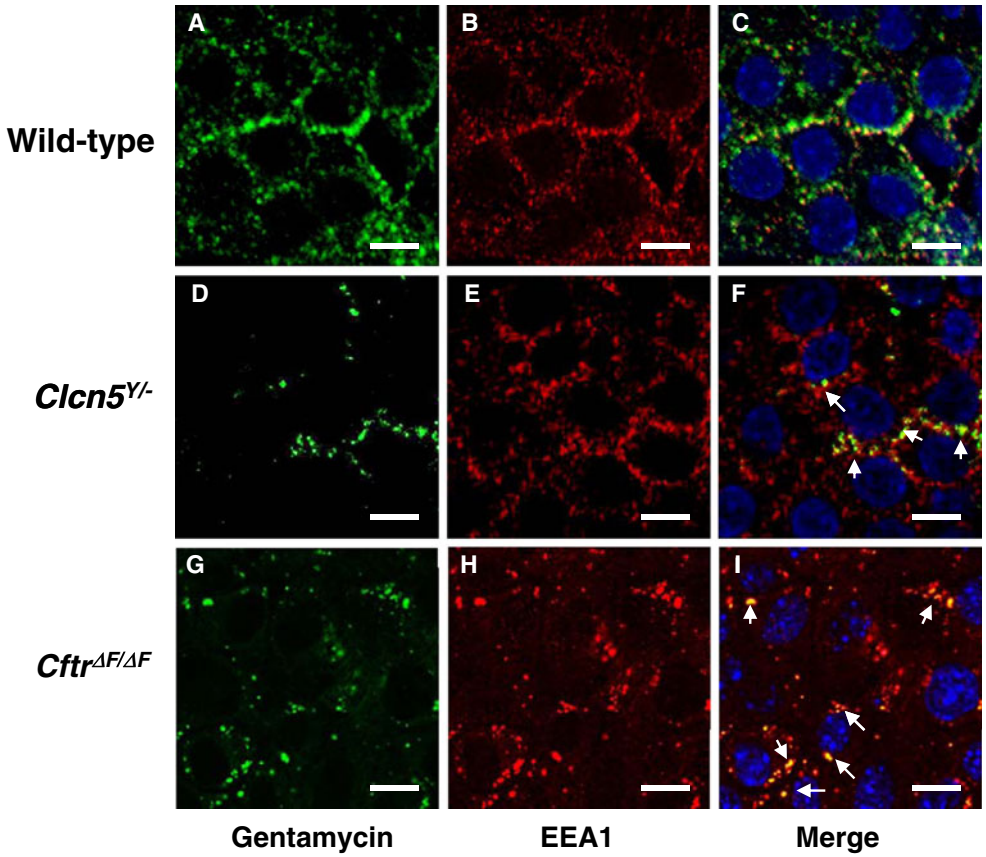

Fig. 3 Early localization of gentamicin in endosomes. Primary cultures of $Clcn5^{Y/+}$ (wild-type, **a**–**c**), $Clcn5^{Y/-}$ (**d**–**f**), and $Cftr^{\Delta F/\Delta F}$ (**g**–**i**) mPTC exposed to 5 mg/mL gentamicin (7 min at 37°C), fixed and stained with anti-gentamicin (Alexa488; **a**, **d**, **g**) and anti-EEA1 (Alexa568; **b**, **e**, **h**). Nuclei counterstained with DraQ5™ (*blue*). Gentamicin colocalizes with EEA1 in multiple vesicles in control mPTC, contrasting with a lower density of vesicles observed in the $Clcn5^{Y/-}$ and $Cftr^{\Delta F/\Delta F}$ cells (Merge panels, *yellow spots*). Scale bar: 10 μm

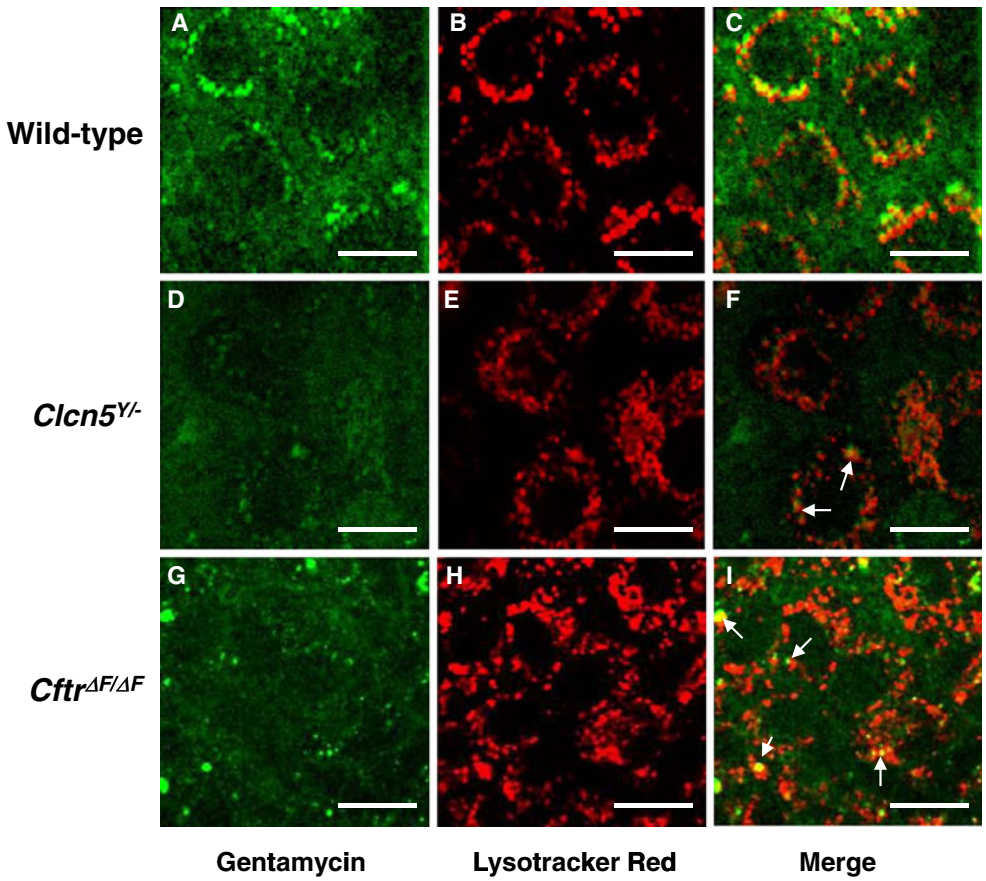

Fig. 4 Late localization of gentamicin in lysosomes. Primary cultures of $Clcn5^{Y/+}$ (wild-type, **a**–**c**), $Clcn5^{Y/-}$ (**d**–**f**), and $Cftr^{\Delta F/\Delta F}$ (**g**–**i**) mPTC exposed to 1 mg/mL Alexa488-gentamicin (*green*; **a**, **d**, **g**) and LysoTracker Red (*red*; **b**, **e**, **h**) for 7 min at 37°C and followed up for 45 min using live-cell imaging. Gentamicin colocalizes with LysoTracker after 45 min (*yellow spots*, merge panels), but a much lower density of positive vesicles was observed in $Clcn5^{Y/-}$ and $Cftr^{\Delta F/\Delta F}$ cells. Scale bar: 5 μm

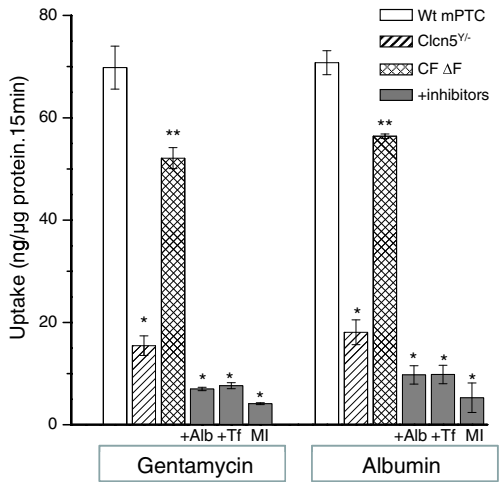

Fig. 5 Quantification of gentamicin uptake in mPTC. Primary cultures of wild-type (Wt) mPTC (*open bars*), $Clcn5^{Y/-}$ (*striped bars*), and $Cftr^{\Delta F/\Delta F}$ (*dashed bars*) mPTC exposed to 0.5 mg/mL Alexa488-gentamicin (*white bars*) or 0.5 mg/mL Alexa488-BSA (*grey bars*) for 15 min at 37°C under control conditions or in the presence of excessive amount of competitive inhibitors albumin (+*Alb*) or transferrin (+*Tf*) or after metabolic inhibition (*MI*). The uptake of albumin and gentamicin is decreased in a variable extent in the $Clcn5^{Y/-}$ vs. the $Cftr^{\Delta F/\Delta F}$ mPTC. The residual uptake after competitive inhibition with an excess of ligands or with metabolic inhibition is shown for comparison. Data are mean±SEM of 6 experiments (n=6). Statistical differences are indicated with *p<0.001 and **p<0.01 vs. Wt mPTC

15 min with similar, non-toxic concentrations of Alexa488-gentamicin or Alexa488-BSA, taken as a well-established ligand for megalin. The uptake of gentamicin or albumin was then analyzed (i) under control conditions, (ii) in the presence of competitive inhibitors of megalin (excess of albumin) or cubilin (excess of transferrin), and (iii) in conditions of metabolic inhibition (Fig. 5). In control mPTC, the amount of accumulated gentamicin was similar to that of albumin (70±4 vs. 71±2 ng/µg protein respectively). Competitive or metabolic inhibition could block gentamicin and albumin uptake by mPTC. As compared to wild-type mPTC, gentamicin and albumin uptake was reduced by ~75% in $Clcn5^{Y/-}$ mPTC and by ~20% in $Cftr^{\Delta F/\Delta F}$ mPTC. These results show that functional loss of ClC-5 or CFTR is reflected by a significant loss in the endocytic uptake of gentamicin (and albumin) by PT cells; this effect is much more pronounced in cells lacking ClC-5 than in those lacking CFTR.

Discussion

In this study, we analyzed the handling of AG by PT cells using in vivo and in vitro experiments performed on $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ mice. Both strains showed a decreased accumulation of gentamicin in the kidney, despite similar levels of renal function and peak gentamicin concentration in the blood. A reduction in the renal uptake of gentamicin was confirmed in mPTC obtained from $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ mice, with much fewer gentamicin-positive vesicles in the early endosomal and lysosomal compartments. The reduction in endocytic uptake was much more pronounced in cells lacking ClC-5 than in those lacking CFTR. These results confirm the role of CFTR in PT cell endocytosis and demonstrate that the functional loss of CFTR, as that of ClC-5, is associated with a decreased efficacy of receptor-mediated uptake in PT cells, causing a decreased renal accumulation of AG.

Among other factors, aggressive antibiotic treatment for lung infections has led to a dramatic increase in life expectancy for patients with CF over the last three decades [25]. Because of their efficacy against *P. aeruginosa*, AG antibiotics are particularly useful to treat chronic lung infections in CF patients [2, 22]. In the general population, up to 25% of treated patients will present AG-induced nephrotoxicity, despite accurate follow-up [14]. The renal toxicity of AG is clearly related to their uptake along the PT segments via receptor-mediated endocytosis [23], accumulation in endo-lysosomal vesicles, modification of the membrane phospholipid content, leakage in the cytosol, and subsequent toxicity for mitochondria and endoplasmic reticulum, activating different pathways leading to cell death [14, 24]. It is known for a long time that CF patients display an enhanced renal clearance for several drugs including AG [13, 26, 30]. Since protein binding is generally unchanged in CF patients and no significant differences in glomerular filtration rate have been found in most studies [26, 30], a possible role for defective endocytic uptake has been evoked. This hypothesis has first been substantiated by Jouret et al., who evidenced a defect in receptor-mediated endocytosis in CF mouse models and CF patients [11]. These studies revealed that CFTR, like ClC-5, is expressed in the endosomes of PT cells [7]. Mice knock-out for CFTR or harboring the F508del mutation presented with a mild but significant albuminuria and LMW-proteinuria, caused by a defective receptor-mediated endocytosis. Patients with CF showed a similar defect in LMW protein handling [11].

To investigate the putative link between defective endocytosis and changes in gentamicin handling in CF, we compared the $Cftr^{\Delta F/\Delta F}$ mouse vs. the $Clcn5^{Y/-}$ mouse model of Dent's disease. The $Cftr^{\Delta F/\Delta F}$ mouse model bears the most common mutation in CF (F508del) [31], which results in misfolding and lack of maturation of CFTR that cannot reach the apical plasma membrane. Instead, the mutated protein does not escape the endoplasmic reticulum quality control and is degraded by the ubiquitin–proteasomal pathway [3]. However, the $Cftr^{\Delta F/\Delta F}$ tissues show a

residual Cl^- conductance, suggesting that the mutant F508del-CFTR is partially processed and reaches the plasma membrane in several tissues including the kidney [19, 31]. This residual activity may explain, at least partially, the less severe PT phenotype in the $Cftr^{\Delta F/\Delta F}$ mouse as compared with the $Clcn5^{Y/-}$ mouse which displays a severe trafficking defect of megalin and cubilin resulting in a massive endocytic defect and generalized PT dysfunction [5, 20, 32]. Our data confirm the quantitative difference in LMW proteinuria and extend it to the renal handling of gentamicin. The decreased renal accumulation of gentamicin in both models could result from a decreased reabsorption as evidenced by immunostaining and quantitative AG analyses in kidney extracts.

Previous studies have identified receptor-mediated endocytosis through binding of megalin as the primary route of uptake for polybasic drugs such as AG [15, 16, 23, 28]. Our in vivo studies show a mild ($Cftr^{\Delta F/\Delta F}$) or dramatic ($Clcn5^{Y/-}$) reduction in megalin staining in PT segments, which could explain the enhanced gentamicin clearance and the decreased renal accumulation. For a more comprehensive investigation of gentamicin handling by PT cells, we used primary cultures of mPTC which remain well differentiated and keep essential properties such as receptor-mediated endocytosis [29]. For instance, we recently showed that the endocytic defect of $Clcn5^{Y/-}$ mice was retained in primary cultured mPTC [21] and that these cells were responding to a transcriptional program of differentiation [27]. Using this system, we demonstrated that the lack of functional CFTR leads to a significant ~20% decrease in gentamicin and albumin uptake, as compared with a major ~80% reduction in cells KO for ClC-5. The fact that similar proportions were obtained for gentamicin clearance and kidney accumulation highlights the correspondence between the distinct methodological approaches targeting the endocytic defect in vivo and in vitro. The sensitivity of gentamicin uptake to metabolic and competitive inhibition (with both excess of albumin and transferrin) identified the energy-demanding receptor-mediated endocytosis pathway using the multiligand receptors megalin and cubilin as the primary uptake route for gentamicin in mPTC.

Our investigation focusing on the trafficking of gentamicin by colocalization with endosomal and lysosomal markers confirmed the AG processing in PT cells. In wild-type mPTC, gentamicin colocalized with EEA1 in the early endosomal pathway after 7-min incubation, whereas most of the gentamicin was localized in lysosomes after 45 min. The gentamicin uptake was reduced in $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ mPTC, but rare colocalization with endosomal and lysosomal markers could be observed. The fact that gentamicin is taken up by receptor-mediated endocytosis and routed to lysosomes opens possibilities to prevent drug accumulation in the PT and thus limiting nephrotoxicity. The search for strategies to prevent the binding of gentamicin to megalin has been initiated [14, 16]. Finally, the different handling of gentamicin observed in the mouse models tested here may suggest that dosage adaptation and drug monitoring is particularly important when using AG in patients suffering from a defective endocytosis and LMW-proteinuria, including CF and Dent's disease.

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