Chapter 19

Segmental and Subcellular Distribution of CFTR in the Kidney

François Jouret, Pierre J. Courtoy, and Olivier Devuyst

Abstract

Besides its location at the plasma membrane, CFTR is present in intracellular vesicles along both the exocytic and the endocytic pathways. *Immunostaining and subcellular fractionation studies* of mouse kidney demonstrate that CFTR is located in endosomes of the cells lining the terminal part of the proximal tubule (PT). The PT cells efficiently reabsorb the ultrafiltered low molecular weight (LMW) proteins by apical endocytosis involving the multiligand receptors megalin and cubilin. The progression from early endosomes to lysosomes depends on the integrity of the cytoskeleton, as well as on vesicular acidification. The latter is mediated by the vacuolar H^+-ATPase (V-ATPase) and requires an anionic conductance to dissipate the transmembrane potential gradient. CFTR might ensure such chloride conductance, thereby participating to endosomal acidification and protein uptake by PT cells. *Immunostaining with well-characterized antibodies* shows that CFTR is located in the terminal segment of PT, where it co-distributes with megalin and cubilin. *Subcellular fractionation* of total mouse kidneys through Percoll gradients demonstrates the co-localization of CFTR with the V-ATPase and early endosome markers including the Cl^-/H^+ exchanger, ClC-5, and the small GTPase, Rab5a. *Deglycosylation studies and immunoblotting* show a distinct glycosylation pattern for CFTR in mouse kidney and lung. The segmental and subcellular distribution of CFTR in mouse kidney supports a role for CFTR in PT receptor-mediated endocytosis of ultrafiltered LMW proteins.

Key words: Cystic fibrosis, CFTR, ClC-5, receptor-mediated endocytosis, megalin, cubilin, endosomal acidification, kidney, proximal tubule cells, low molecular weight protein, antigen retrieval, immunostaining, analytical subcellular fractionation, Percoll gradients.

1. Introduction

A significant amount of albumin and low molecular weight (LMW) plasma proteins is continuously filtered through the glomeruli, to be reabsorbed by proximal tubule (PT) cells (1). By

M.D. Amaral, K. Kunzelmann (eds.), *Cystic Fibrosis*, Methods in Molecular Biology 741,
DOI 10.1007/978-1-61779-117-8_19,

definition, LMW proteins are characterized by a molecular mass lower than that of albumin (69 kDa). Most filtered LMW proteins are reabsorbed and metabolized by PT cells, and the human urine is virtually devoid of plasma proteins under physiological conditions. This massive uptake of proteins from the primitive urine accounts for as much as 80% of the total metabolic clearance of small proteins and peptides and plays a key role in hormone and vitamin homeostasis (for a review, see (2)).

The uptake of LMW proteins by PT cells essentially involves receptor-mediated endocytosis. Clathrin-mediated endocytosis represents the predominant pathway for protein uptake across the apical membrane of PT cells, with an endocytic pathway consisting of five main interrelated compartments: (i) microvilli and clathrin-coated pits, (ii) early endosomes, (iii) dense apical tubules responsible for apical recycling, (iv) late endosomes, and (v) lysosomes (3). The process essentially requires two multiligand receptors, megalin and cubilin, that are expressed at the brush border of PT cells (2). Ligand binding and interactions between both receptors induce their internalization into coated vesicles and their subsequent delivery to endosomes and lysosomes for ligand processing and receptor degradation or recycling (**Fig. 19.1**). Receptor-mediated endocytosis of albumin depends on the integrity of the actin cytoskeleton and the microtubules (4), whereas progression along the endocytic apparatus requires a sustained vesicular acidification from early to late endosomes and finally to lysosomes (5, 6). In PT cells, the endosomal acidification is driven by the electrogenic vacuolar H^+-ATPase. The translocation of H^+ from the cytoplasm into the endosomes generates a transmembrane electrical potential ($\Delta\Psi$) resulting in a rapid inhibition of V-ATPase activity. Thus, in order to ensure progressive vesicular acidification, either anions have to enter vesicles or cations have to leave. In most cases, vesicular acidification seems dependent on a parallel Cl^- conductance (7, 8). Furthermore, the intravesicular Cl^- concentration itself could directly affect the V-ATPase activity (9) as well as the vesicle recycling independently of its effect on pH (6).

Besides its location at the plasma membrane, CFTR is distributed in intracellular organelles along the endocytic and secretory pathways, in which it might act as a pH regulator by importing Cl^- in parallel to H^+ accumulation (10). Mutant epithelial cells derived from CF patients exhibit no cAMP-dependent regulation of endocytosis and exocytosis, unless transfected with cDNA encoding wild-type CFTR (11). Incubation of freshly isolated nasal polyps from CF patients harboring the F508del mutation with 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP), used as a semiquantitative marker of vesicular acidification, showed that DAMP accumulation was significantly lowered in specific biosynthetic compartments, i.e.,

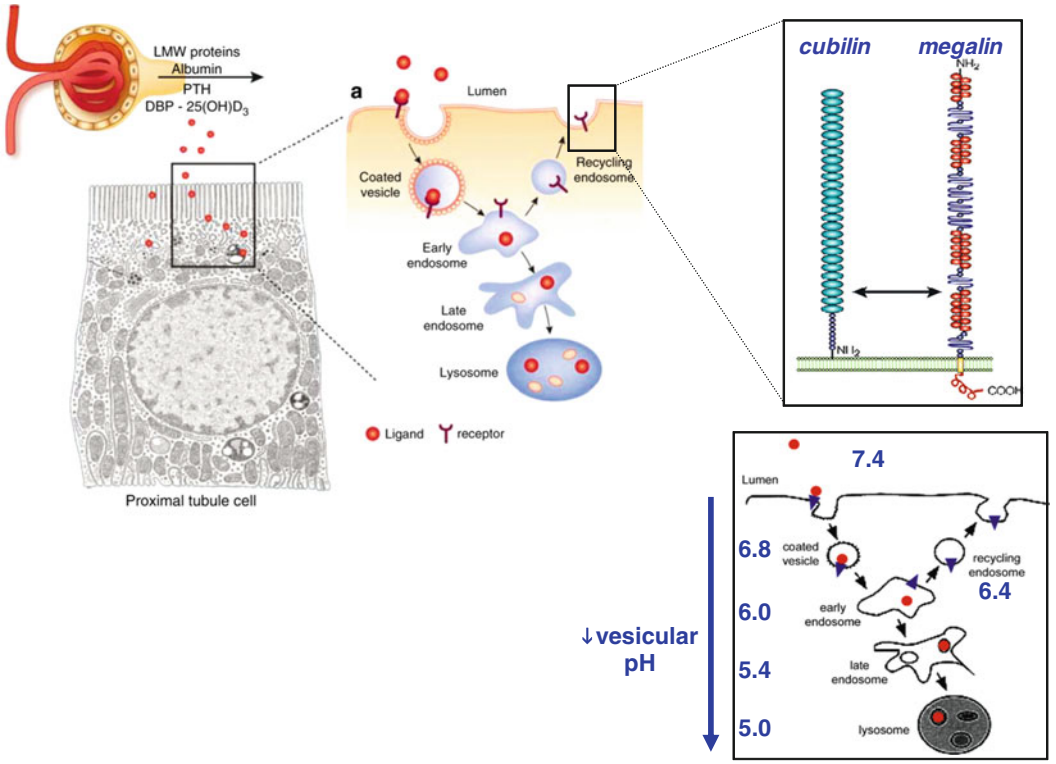

Fig. 19.1. **Reabsorption of low molecular weight proteins by proximal tubule cells.** Albumin and low molecular weight (LMW) proteins that are naturally filtered by the glomerulus into the primary urine are endocytosed by PT cells via the megalin/cubilin receptor pathway. Following internalization in coated vesicles, the receptor–ligand complexes progress along the endocytic pathway. The endosomes undergo a progressive acidification that results in the dissociation of the receptor–ligand complexes, with megalin and cubilin (*inset*) being recycled to the apical membrane, whereas the ligand is directed to lysosomes for degradation. Modified from (18).

trans-Golgi and pre-lysosomal organelles (12). Moreover, the monitoring of membrane potential in a light microsomal fraction from CF and non-CF epithelial cells showed that acidification is limited in CF cells by a high $\Delta\Psi$ resulting from insufficient Cl^- counterion conductance. In turn, defective acidification may induce lysosomal enzyme deficiencies and abnormal trafficking and processing of newly synthesized polypeptides in cells lacking CFTR (10). However, the role of CFTR in regulating organelle pH remains controversial, with hyper- rather than hypo-acidification suggested to occur in CF respiratory epithelial cells. Indeed, cell ratiometric imaging with luminally exposed pH-sensitive green fluorescent protein has demonstrated that CFTR decreases the pH of endosomal organelles because of a loss of CFTR inhibitory effects on Na^+ transport and a defect in cyclic guanosine 3,5-monophosphate signaling cascade (13). In addition, recycling of transferrin receptor is impaired in CFTR mutant lung epithelial cells, with possible functional consequences at the plasma membrane and within endosomal compartments (14).

This section focuses on the segmental and subcellular distribution of CFTR in the epithelial cells lining the PT of mouse kidney using immunostaining following antigen retrieval, as well as immunoblotting and analytical subcellular fractionation through Percoll gradients. These techniques defined the distribution and role of CFTR in the endocytic pathway operating in PT cells. Further biochemical and functional investigations performed on CFTR-deficient mouse models and in CF patients have confirmed the role of CFTR in the uptake of ultrafiltered LMW proteins by the mammalian kidney (15).

2. Materials

2.1. Antigen Retrieval and Immunoperoxidase Labeling Procedures with Paraffin-Embedded Serial Sections of Mouse Kidney

2.1.1. Formaldehyde Solution

1. Phosphate buffer (0.2 M, pH 7.4):
   (a) Solution A: dilute 22.7 g Na_2HPO_4 anhydrous (Sigma S3397, Sigma-Aldrich, St. Louis, MO) in 800 ml of double distilled water (DDW)
   (b) Solution B: dilute 6.2 g KH_2PO_4 (Sigma P9791) in 250 ml DDW
   (c) Titrate the pH of solution A up to 7.4 by adding solution B
   (d) Level it up to 1 l with DDW
2. Dilute 4 g paraformaldehyde in phosphate buffer (0.2 M, pH 7.4)
3. Make fresh as required
4. Wear a mask when working with formaldehyde powder

2.1.2. Citrate Buffer

1. Mix in 1 l of DDW:
   (a) Citric acid $C_6H_8O_7$ H_2O (Merck 244, Overijse, Belgium): 3.77 g
   (b) Sodium citrate $C_6H_5Na_3O_7$ $2H_2O$ (Merck 1.06448.0500): 25.12 g
2. Store the solution (1 M, pH 5.8) as aliquots at –20°C for up to 3 months
3. Make fresh the final solution (0.01 M), as required

2.1.3. Ethanol Solutions at Distinct Concentrations (Dilute with DDW)

Dilute ethanol with DDW into decreasing concentrations: 100–96–80–70–50–30%.

2.1.4. Methanol-H_2O_2 (0.3 %) Solution

1. Add 2 ml H_2O_2 (Sigma H1009, stable at 4°C for up to 1 month) to 198 ml methanol
2. Make it fresh as required

2.1.5. TRIS Solution (1 M, pH 7.4)

1. Mix in 2 l of DDW
   (a) TRIZMA base (Sigma T6066): 12.2 g
   (b) NaCl: 50 g
   (c) HCl 32%: 8.75 ml
2. Titrate up to pH 7.4

2.1.6. TRIS-Tween 0.02 %

1. Add 160 µl of Tween-20 (Sigma P1379) to 800 ml TRIS solution

2.1.7. TRIS-Bovine Serum Albumin (BSA) 2 %

1. Add 2 g of BSA (Sigma A-7638) to 100 ml TRIS solution

2.1.8. ABC Vectastain Elite (Vector Laboratories, Orton Southgate, Peterborough, UK)

2.1.9. 3-Amino-9-Ethylcarbazole (AEC) Reagent Kit (Vector Laboratories)

2.2. Analytical Subcellular Fractionation and Immunoblotting

2.2.1. Anesthetic Drugs

1. Rompun® (40 µg/g body weight; stock 2%; Bayer, Brussels, Belgium)
2. Anesketin® (20 µg/g body weight; Eurovet, AD Bladel, The Netherlands)

2.2.2. SIPPI Solution

1. Sucrose (Sigma S7903) 0.25 M
2. Imidazole (Sigma I-0125) buffer 3 mM, pH 7.4
3. Protease inhibitors (Roche, Brussels, Belgium): 1 pellet/50 ml

4. Phosphatase inhibitors (sodium orthovanadate 2 mM, Acros Organics, Geel, Belgium; NaF 10 mM, Merck)

2.2.3. Percoll (Sigma P1644)

2.2.4. Sample Buffer (5x)

1. Mix in 50 ml of DDW:
   (a) SDS (Lauryl sulfate, Invitrogen 15525-017, Merelbeke, Belgium): 3.75 g
   (b) Glycerol (Sigma G8773): 15 ml
   (c) Bromophenol blue 0.1% (Sigma B8026): 2 ml
   (d) TRIS (Bio-Rad 161-0719) 1 M, pH 6.8: 2.5 ml
2. Keep at 4°C (to avoid SDS precipitation)

2.2.5. Fairbanks Buffer (20x)

1. Mix in 1 l of DDW
2. TRIZMA base (Sigma T6066): 48.19 g
3. Sodium acetate: 16.41 g (Sigma S3272)
4. Na-EDTA: 7.44 g (Sigma E5513)
5. Titrate to pH 7.4 with acetic acid
6. Make it fresh as required by diluting 20 times with distilled water

2.2.6. Block Buffer

1. Mix in 800 ml of DDW
   (a) Na_3PO_4 (Aldrich 342483) (0.5 M, pH 7.4): 80 ml
   (b) NaCl 5 M: 24 ml
   (c) Tween-20 (Sigma P1379): 8 ml

2.2.7. Milk Block Buffer

1. Dilute 2 g milk powder 5% in 40 ml Block buffer

3. Methods

3.1. Antigen Retrieval and Immunoperoxidase Labeling Procedures with Paraffin-Embedded Serial Sections of Mouse Kidney

3.1.1. Paraffin Removal and Rehydration

1. Paraffin removal: immerse the slides into xylene for 4 min.
2. Rehydration: immerse the slides for 3 min into ethanol solutions of decreasing concentration (100–96–80–70–50–30%). Rinse for 5 min in DDW.

3.1.2. Antigen Retrieval (See Note 1)

1. Immerse the slides into citrate buffer pre-warmed to 97°C for 75 min.
2. Let the slides in solution cool down at room temperature for 20 min.
3. Rinse the slides with DDW for 5 min and prepare the methanol-H_2O_2 (0.3%) solution.

3.1.3. Inhibition of Endogenous Peroxidase

1. Immerse the slides into methanol-H_2O_2 (0.3%) solution at room temperature for 30 min.
2. Rinse the slides with TRIS solution (2 × 10 min).

3.1.4. Block of Unspecific Immunoreactive Sites (See Note 2)

1. Prepare the blocking solution by diluting 75 µl blocking serum (non-immune serum of same species as primary or secondary antibodies) into 5 ml TRIS solution. Vortex and keep it on ice.
2. Dry the slides individually with paper and/or aspiration.
3. Delineate the tissue section with the hydrophobic pen (5 mm far from the sample).
4. Place the slide on a support in a humid chamber.
5. Drop 150 µl blocking serum on tissue samples.
6. Incubation time: 20 min.

3.1.5. Incubation with Primary Antibodies (See Note 3)

1. Dilute the primary antibodies in an Eppendorf with TRIS-BSA 2% solution. Keep it on ice.
   (a) Affinity-purified rabbit polyclonal antibodies (MD1314) against the C-terminus of rodent CFTR (MD1314, Dr. C.R. Marino, VA Medical Center, University of Tennessee, Memphis, TN): 1/500;
   (b) Rabbit polyclonal antibodies against the water channel, AQP1 (Chemicon, Temecula, CA): 1/400.
2. Aspirate the blocking serum and drop the solution with primary antibodies on tissue samples.
3. Incubation time: 45 min.
4. Save the primary antibodies for future experiments.
5. Rinse with TRIS-Tween (2 × 5 min) and TRIS (5 min).

3.1.6. Incubation with Secondary Biotinylated Antibodies

1. Mix in an Eppendorf 5 ml TRIS solution, 75 µl blocking serum, and 25 µl secondary biotinylated antibodies (Vector Laboratories, Brussels, Belgium; final concentration: 1/200). Vortex and keep it on ice.
2. Prepare the slides as described in **Section 3.1.4**.
3. Drop 150 µl of the solution with secondary antibodies on tissue samples.

4. Incubation time: 45 min.
5. Rinse with TRIS-Tween (2 × 5 min) and TRIS (5 min).

3.1.7. Incubation with Avidin-Coupled Peroxidase

1. Dilute the avidin DH/biotinylated horseradish peroxidase H macromolecular complex (Vectastain Elite ABC kit PK6100, Vector Laboratories) in TRIS solution (5 ml).
2. Prepare the slides as described in **Section 3.1.4**.
3. Drop 150 µl of the ABC solution on tissue samples.
4. Incubation time: 45 min.
5. Rinse with TRIS (3 × 5 min).

3.1.8. Revelation Using the 3-Amino-9-Ethylcarbazole (AEC) Reagent Kit (Vector Laboratories, SK4200)

1. Immediately before use, prepare the substrate solution according to the manufacturer's recommendations:
   (a) Add 2 drops of the Buffer Stock solution to 1 ml distilled water;
   (b) Add 3 drops of the AEC Stock solution and mix;
   (c) Add 2 drops of the hydrogen peroxide solution and mix.
2. Prepare the slides as described in **Section 3.1.4**.
3. Drop 150 µl of the final AEC solution on tissue samples.
4. Incubation time: 15 min.
5. Rinse with distilled water (3 × 5 min).

3.1.9. Mounting of Slides (See Note 4)

1. Aspire the liquid completely from the slide and mount them with glycerol-enriched water.
2. Leave the slide on a flat surface for the next 24 h before viewing.

3.1.10. Imaging Procedure

1. Use a Leica DMR coupled to a Leica DC300 digital camera (Leica, Heerbrugg, Switzerland) (**Fig. 19.2**)

3.2. Analytical Subcellular Fractionation and Immunoblotting Procedures

3.2.1. Tissue Sampling (See Note 5)

1. Measure the mouse body weight.
2. Induce anesthesia by intraperitoneal injection of Rompun® (40 µg/g body weight; stock 2%; Bayer, Brussels, Belgium) and Anesketin® (20 µg/g body weight; Eurovet, AD Bladel, The Netherlands).
3. Inject ^{125}I-β_2-microglobulin (M4890, Sigma-Aldrich) in the periorbital plexus (typically >1,000 cpm/ng and >95% trichloroacetic acid precipitable; ~600 ng radioligand per

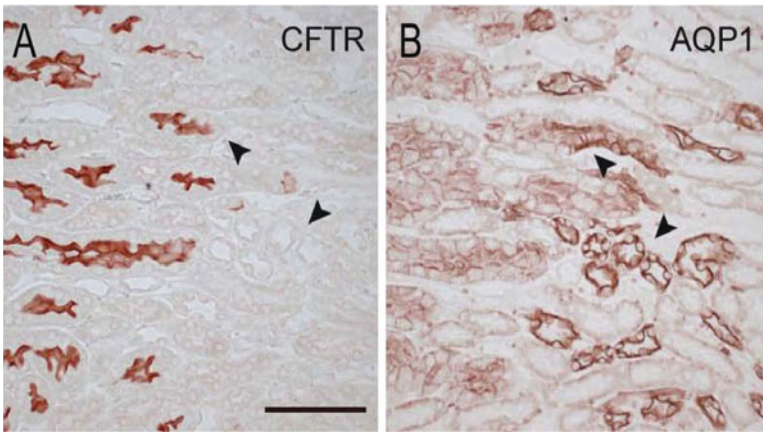

Fig. 19.2. **Segmental distribution of CFTR along the mouse nephron.** Immunoperoxidase labeling for CFTR (**a**) and AQP1 (**b**) was performed on serial sections (5 μm) of mouse *Cftr*$^{+/+}$ kidneys. The high signal for CFTR at the cortico-medullary junction, combined with the segmental co-localization of CFTR and AQP1, indicates that CFTR is particularly abundant in the apical area of the distal S3 segment of PT, just before the transition with the descending thin limb (**a** and **b**, *arrowheads*). Bar: 100 μm.

gram body weight injected in a maximal volume of 150 μl; the exact dose is calculated by weighing the syringe before and after injection).

4. Perform laparotomy, expose kidneys and *vena cava inferior*. Section the vein.
5. Collect a small sample of blood with a heparinized tuberculin syringe for further analysis.
6. Flood with PBS kept at 37°C until no blood efflux can be seen (~20 s).
7. Excise exsanguinated kidneys, section the hile, and remove kidney capsule.
8. From this point, all subsequent steps are performed at 4°C.
9. Weigh kidneys in ice-cold SIPPI.
10. If fractionation is combined with autoradiography, ~1/3 of one kidney is fixed by immersion in cold, 4% (w/v) freshly prepared formaldehyde overnight, followed by rinsing in cold PBS and paraffin embedding.

3.2.2. Homogenization and Differential Sedimentation

1. Mince kidneys and homogenize in SIPPI with three to five strokes of a Potter–Elvehjem tissue homogenizer (B1804, Thomas Scientific, Swedesboro, NJ).

2. Perform a first low-speed sedimentation at 10,000×*g* × 10 min (Jouan centrifuge; 2,070 rpm, 4°C) and collect the first low-speed supernatant.
3. Extract again the low-speed pellet by rehomogenization using three strokes of the Potter–Elvehjem homogenizer and centrifuge again at 10,000×*g* × 10 min; the final pellet, denoted nuclear fraction ("N"), corresponds to nuclei, cell debris, and insoluble extracellular matrix.
4. Pool low-speed supernatants as "post-nuclear supernatant" (referred to as "MPLS") to a final volume of ~8 ml; save 1 ml for further analysis.
5. Submit the remaining 7 ml to high-speed centrifugation at 100,000×*g* × 60 min (Ti50 rotor, Beckman, Palo Alto, CA; 40,000 rpm), using acceleration and brake (Optima LE-80 K ultracentrifuge, Beckman).
6. The high-speed supernatant (~6.5 ml), denoted fraction "S," corresponds to the cytosol; the high-speed pellet corresponds to post-nuclear particles (referred to as "MLP" fraction). Resuspend this MLP fraction with a tigette in ~2 ml of SIPPI and further homogenize with a Dounce (loose pestle). One milliliter is used for further analysis by density gradient centrifugation, and the remainder is saved for further analysis.

3.2.3. Density Gradient Centrifugation (See Note 6)

1. Verify that organelles are well homogenized as individual particles by evidence of Brownian motion under phase contrast microscopy.
2. Mix 1 ml of post-nuclear particles with 7 ml of Percoll at 16% (v/v) in a #355630 centrifuge tube (Beckman); the heterogeneity (polydispersity) of Percoll particles will ensure a self-generating gradient upon centrifugation (in this case, close to linearity).
3. Inject 250 µl of pure Percoll below this mixture using a syringe equipped with a long needle, as cushion.
4. Resolve subcellular particles in this self-forming gradient by centrifugation at 60,000×*g* × 30 min (30,000 rpm in the Ti50 rotor).
5. Withdraw the brake shortly before the end of centrifugation, when speed declines to ~1,000 rpm.
6. Collect 10 fractions (of ~800 µl each) in pre-weighed empty tubes, starting by aspiration of the bottom of the tube.
7. Weigh filled tubes and determine the fraction weight by the difference.
8. Determine density refractometry (Refractometer Abbé, Bellingham and Stanley). The exact volume of each fraction is given by the ratio of weight to density.

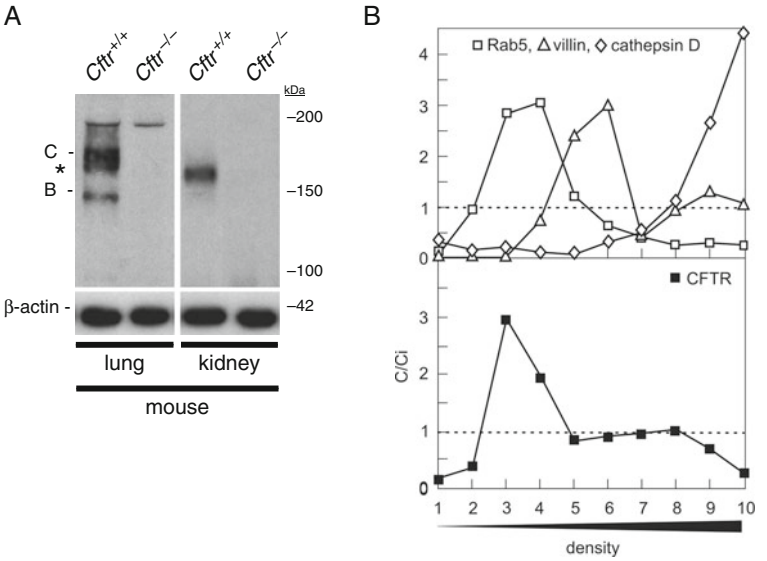

Fig. 19.3. **Immunoblotting and density distribution of CFTR in mouse kidney homogenates using Percoll gradient.** (**a**) Representative immunoblotting for CFTR in lung and kidney from *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice. Membrane extracts (30 μg/lane) were run on 5% PAGE and transferred to nitrocellulose. The blot was probed with MD1314 anti-CFTR antibodies (1:500) and, after stripping, β-actin (1:10,000). In lung, both B- (150 kDa) and C-bands (180 kDa) are present, whereas, in kidney, CFTR is detected as a single large band at approximately 160 kDa (*asterisk*). In both tissues, immunoreactive bands for CFTR are absent in *Cftr*$^{-/-}$ extracts. (**b**) Distributions after centrifugation are presented by comparison with the initial concentration as C/Ci (*broken line*), where values >1 reflect organelle enrichment and values <1 reflect organelle depletion. The *upper panel* indicates the resolution of endosomal peak (Rab5a), brush border (villin), and lysosomes (mature cathepsin D). The *lower panel* indicates that CFTR peaks at the position of endosomes, is not enriched at the position of the brush border, and is depleted at the position of lysosomes. The shape of the gradient is given by the density as indicated below. Whereas the positions of endosomes and lysosomes are constant, the position of the brush border varies according to slight variations of the shape of the Percoll gradient (for additional information, see (15, 19–20)).

3.2.4. Distribution Analysis (See Note 7)

1. Count radioactivity (e.g., Minaxi Auto Gamma 5,000 series, Gamma counter, Packard) in dilutions corresponding to 5 μl of injection or 10 μl of blood, in 100 μl of differential sedimentation fractions (nuclear fraction, post-nuclear supernatant, post-nuclear particles, and high-speed supernatant) and 250 μl for each fraction of the Percoll gradient.
2. Probe fractions by immunoblotting for organelle markers (**Fig. 19.3**).

3.2.5. Immunoblotting

1. *Handling of the samples* (*see* **Note 8**)
   (a) Aliquot each fraction obtained through the Percoll gradient (from #1 to #10) and add sample buffer as 4/1 (v/v).
   (b) Heat the samples at 60°C for 12 min.

(c) Place 10 μl of each sample in individual pits at the top of 0.1 × 9 × 6 cm SDS 5%-polyacrylamide gel (PAGE) slabs.

2. *Migration of the samples through the SDS-PAGE* (*see* **Note 9**)
   (a) Start the migration with a potential difference of 100 V for 10 min.
   (b) Increase the potential to 200 V once the samples have fully entered the gel. Keep it for at least 40 min.
3. *Transfer of the samples from the SDS-PAGE to the nitrocellulose membrane* (*see* **Note 10**)
   (a) Immerse the SDS-PAGE and the nitrocellulose membrane between two fiber pads in Fairbanks. Cool the solution down to 4°C by setting ice pack in the container.
   (b) Turn it on, with a current of 250 mA for 60 min (electroblotting).
   (c) Remove the membrane from the SDS-PAGE and identify the molecular weight standards with a blue pen.
   (d) Wash the membrane with distilled water.
4. *Block of aspecific immunoreactive sites*
   (a) Incubate the membrane with Milk Block buffer for 30 min.
5. *Incubation with primary antibodies* (*see* **Note 11**)
   (a) Dilute the primary antibodies in Block buffer 2% BSA:
      - Affinity-purified rabbit polyclonal antibodies (MD1314) against the C-terminus of rodent CFTR (Dr. C.R. Marino): 1/1,500;
      - Rabbit polyclonal antibodies against Rab5a (KAP-GP006, Stressgen): 1/5000;
      - Rabbit polyclonal antibodies against villin (Dr. D. Louvard and S. Robine, Institut Curie, Paris, France): 1/10,000;
      - Goat polyclonal antibodies against mature cathepsin D (C-20, SC6486, Santa Cruz Biotechnology): 1/2,000.
   (b) Remove the Milk Block buffer.
   (c) Wash the membrane with Block buffer for 5 min.
   (d) Incubate the membrane overnight at 4°C with primary antibodies.
6. *Incubation with secondary antibodies*
   (a) Save the primary antibodies for future experiments.
   (b) Rinse with Block buffer for 15 min (2×) and 5 min (2×).

(c) Incubate the nitrocellulose membrane with secondary antibodies (dilution: 1/1,500 in Milk Block) at room temperature for 60 min.

7. ***Chemiluminescence detection***
   (a) Wash the membrane with Block buffer for 15 min (2×) and 5 min (2×).
   (b) Incubate the nitrocellulose membrane with Pierce ECL solution (Thermo Fisher Scientific, Erembodegem, Belgium).
   (c) Dry the membrane and expose to X-ray film for 1–5 min (**Fig. 19.3**)

4. Notes

1. Make sure that citrate buffer has been pre-warmed to 97°C in a water bath. Do not use a microwave oven to warm your solutions and slides because of the lack of homogeneity in the heating. Plunging the container filled with citrate buffer allows a homogenous and straight heating (up to 97°C).
2. Do not touch tissue samples during manipulations. Tissue samples may not dry. Thus, manipulate the slides one by one and leave the others in TRIS solution (or in the humid chamber). Make sure that the blocking serum covers the entire surface which has been delineated by the hydrophobic pen.
3. Make sure that primary antibodies have been correctly thawed before starting the procedure. Prepare enough solution to cover each circled area with ~150 μl, but retrieve precious antibodies after use. Take advantage of each rinsing period to prepare the next solutions.
4. AEC produces a red reaction product. Slides developed with AEC must be mounted in a water solution since the reaction product is soluble in organic solvents. Monitor (by eye) the incubation time, which may vary from 10 to 30 min.
5. Keep a sample from each animal in order to confirm genotype. For example, cut the tip of mouse tail, identify it, and freeze it in an individual plastic bag.
6. The verification that organelles are well homogenized by evidencing Brownian motion of individual particles under phase contrast microscopy is essential to exclude aggregation.
7. When interpreting data, remember that (i) distributions must be validated by recovery (sum of all fractions =

starting material ± 20% max) and resolution (separation between peaks) and (ii) separation is proof, co-distribution may also reflect aggregation (16).

8. Use specific loading tip to place each sample into the defined lane at the top of the gel. Be careful not to contaminate adjacent fractions when loading. One empty lane is advised between MLP and gradient fractions.
9. Check the correct migration of the colored molecular weight standards in order to avoid loss of target proteins going out of the gel.
10. Check transfer efficiency by incubating the nitrocellulose membrane with Ponceau Red dye (Sigma) for 1 min: sample smear should rapidly appear.
11. When characterizing the position of lysosomes in Percoll gradients by probing the fractions by immunoblotting, do *not* rely on LAMP-1, which is associated with both lysosomes and low-density endosomes. Prefer the distribution of mature cathepsin D. Alternatively assay for *N*-acetyl-β-hexosaminidase activity, which is *not* inhibited by protease and phosphatase inhibitors.

Acknowledgments

The authors thank Y. Cnops, Th. Lac, and P. Van der Smissen for excellent technical assistance. The *Cftr* mice were kindly provided by H. R. De Jonge (Erasmus University Medical Center, Rotterdam, The Netherlands) and the anti-CFTR antibody MD1314 by C.R. Marino (University of Tennessee, Memphis, TN).

P. Courtoy wishes to thank G. Dom, M. Leruth, B. Marien, and F. N'Kuli for patiently adapting to mouse kidney the analytical subcellular fractionation procedure originally developed for rat liver (17). These studies were supported by the Belgian agencies FNRS and FRSM, the "Fondation Alphonse and Jean Forton," Concerted Research Actions, an Inter-university Attraction Pole (IUAP P6/05), the DIANE project (Communauté Française de Belgique), and the EUNEFRON (FP7, GA#201590) program of the European Community.

References

1. Birn, H., and Christensen, E. I. (2006) Renal albumin absorption in physiology and pathology. *Kidney Int.* **69**, 440–449.
2. Christensen, E. I., and Birn, H. (2002) Megalin and cubilin: multifunctional endocytic receptors. *Nat. Rev. Mol. Cell. Biol.* **3**, 256–266.
3. Conner, S. D., and Schmid, S. L. (2003) Regulated portals of entry into the cell. *Nature* **422**, 37–44.

4. Gekle, M. (2005) Renal tubule albumin transport. *Annu. Rev. Physiol.* **67**, 573–594.
5. Shi, L. B., Fushimi, K., Bae, H. R., and Verkman, A. S. (1991) Heterogeneity in ATP-dependent acidification in endocytic vesicles from kidney proximal tubule. Measurement of pH in individual endocytic vesicles in a cell-free system. *Biophys. J.* **59**, 1208–1217.
6. Faundez, V., and Hartzell, H. C. (2004) Intracellular chloride channels: determinants of function in the endosomal pathway. *Sci. STKE* **233**, re8.
7. Jentsch, T. J. (2008) CLC chloride channels and transporters: from genes to protein structure, pathology and physiology. *Crit. Rev. Biochem. Mol. Biol.* **43**, 3–36.
8. Devuyst, O., and Guggino, W. B. (2002) Chloride channels in the kidney: lessons learned from knockout animals. *Am. J. Physiol. Renal Physiol.* **283**, F1176–F1191.
9. Moriyama, Y., and Nelson, N. (1987) The purified ATPase from chromaffin granule membranes is an anion-dependent proton pump. *J. Biol. Chem.* **262**, 9175–9180.
10. Bradbury, N. A. (1999) Intracellular CFTR: localization and function. *Physiol. Rev.* **79**, S175–S191.
11. Bradbury, N. A., Jilling, T., Berta, G., Sorscher, E. J., Bridges, R. J., and Kirk, K. L. (1992) Regulation of plasma membrane recycling by CFTR. *Science* **256**, 530–532.
12. Barasch, J., Kiss, B., Prince, A., Saiman, L., Gruenert, D., and Al-Awqati, Q. (1991) Defective acidification of intracellular organelles in cystic fibrosis. *Nature* **352**, 70–73.
13. Poschet, J. F., Skidmore, J., Boucher, J. C., Firoved, A. M., Van Dyke, R. W, and Deretic, V. (2002) Hyperacidification of cellubrevin endocytic compartments and defective endosomal recycling in cystic fibrosis respiratory epithelial cells. *J. Biol. Chem.* **277**, 13959–13965.
14. Poschet, J. F., Fazio, J. A., Timmins, G. S., Ornatowski, W., Perkett, E., Delgado, M., et al. (2006) Endosomal hyperacidification in cystic fibrosis is due to defective nitric oxide-cyclic GMP signalling cascade. *EMBO Rep.* **7**, 553–559.
15. Jouret, F., Bernard, A., Hermans, C., Dom, G., Terryn, S., Leal, T., et al. (2007) Cystic fibrosis is associated with a defect in apical receptor-mediated endocytosis in mouse and human kidney. *J. Am. Soc. Nephrol.* **18**, 707–718.
16. Beaufay, H., and Amar-Costesec, A. (1976) Cell fractionation techniques. In (Korn, E. D., ed.) *Methods in Membrane Biology*, vol. 6. Plenum Press, New York, NY, pp. 1–100.
17. Courtoy, P. J. (1993) Analytical subcellular fractionation of endosomal compartments in rat hepatocytes. In (Bergeron, J. J. M., Harris, J. R., eds) *Subcellular Biochemistry: Endocytic Components: Identification and Characterization*, vol. 19. Plenum Press, New York, NY, pp. 29–68.
18. Devuyst, O., and Pirson, Y. (2007) Genetics of hypercalciuric stone forming diseases. *Kidney Int.* **72**, 1065–1072.
19. Draye, J.-P., Courtoy, P. J., Quintart, J., and Baudhuin, P. (1987) Relations between plasma membrane and lysosomal membrane. 2. Quantitative evaluation of plasma membrane marker enzymes in the lysosomes. *Eur. J. Biochem.* **170**, 405–411.
20. Christensen, E. I., Devuyst, O., Dom, G., Nielsen, R., Van der Smissen, P., Verroust, P., et al. (2003) Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. *Proc. Natl. Acad. Sci. USA* **100**, 8472–8477.