Blue Native/SDS-PAGE Analysis Reveals Reduced Expression of the mClCA3 Protein in Cystic Fibrosis Knock-out Mice*

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Cystic fibrosis (CF) is a frequent autosomal recessive disorder caused by mutation of a gene encoding a multifunctional transmembrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR), located in the apical membrane of epithelial cells lining exocrine glands. In an attempt to get a more complete picture of the pleiotropic effects of the CFTR defect on epithelial cells and particularly on the membrane compartment, a bidimensional blue native (BN)/SDS-PAGE-based proteomic approach was used on colonic crypt samples from control and CFTR knock-out mice (cfr−/−). This approach overcomes the difficulties of membrane protein analysis by conventional two-dimensional PAGE and is able to resolve multiprotein complexes. Used here for the first time on crude membrane proteins that were extracted from murine colonic crypts, BN/SDS-PAGE allows effective separation of protein species and complexes of various origins, including mitochondria, plasma membrane, and intracellular compartments. The major statistically significant difference in protein maps obtained with samples from control and cfr−/− mice was unambiguously identified as mClCA3, a member of a family of calcium-activated chloride channels considered to be key molecules in mucus secretion by goblet cells. On the basis of this finding, we evaluated the overall expression and localization of mClCA3 in the colonic epithelium and in the lung of mice by immunoblot analysis and immunohistochemistry. We found that mClCA3 expression was significantly decreased in the colon and lung of the cfr−/− mice. In an ex vivo assay, we found that the Ca2+-dependent (carbachol-stimulated) glycoprotein secretion strongly inhibited by the calcium-activated chloride channel blocker niflumic acid (100 μM) was impaired in the distal colon of cfr−/− mice. These results support the conclusion that a Cl−/Ca2+ exchange function in the CF colon depends on CFTR expression and may be correlated with the impaired expression of mClCA3. Molecular & Cellular Proteomics 4:1762–1775, 2005.

Cystic fibrosis (CF) is an autosomal recessive disorder associated with multisystem complications that especially compromise intestinal, pancreatic, and pulmonary functions. The underlying cause of CF is the presence of mutations in the CFTR gene that lead to defective functions of the corresponding cystic fibrosis transmembrane conductance regulator (CFTR), a 1480-amino acid transmembrane protein principally located in the apical membrane of cells lining exocrine glands, including lung, sinus, pancreas, intestine, sweat ducts, and vas deferens.

Although the CFTR protein is an ATPase/ATP-binding cassette transporter that functions as a cyclic AMP and protein kinase A-activated anionic channel, it also exerts regulatory functions over various transport proteins, e.g. CFTR inhibits the apical sodium transporter ENaC (epithelial Na+ channel) and activates the outwardly rectifying chloride channel (ORCC) (1, 2).

In CF epithelia, CFTR deficiency is thought to result in defective anion secretion and excessive Na+ reuptake across epithelia, leading to insufficient intraluminal hydration, which causes mucus accumulation, and defective impaction states in intestine as well as impaired mucociliary clearance in airways. Nevertheless this model of pathogenesis, based on the principle of CFTR-controlled fluid homeostasis, is not a wholly satisfactory paradigm because it does not explain how the CFTR defect can give rise to the multiplicity of abnormalities that have been associated with the disease. These include changes in levels of protein secretion, in post-translational modifications of the secreted proteins, in intravesicular acidification, and in inflammatory and innate immune responses (1, 2).

1 The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; cfr−/− and cfrtm1UNC, cfr knock-out mice; BN, blue native; 2D, two-dimensional; CaCC, calcium-activated chloride channel; NA, niflumic acid; BisTris, 2-[bis[2-hydroxyethyl]amino]-2-[hydroxyethyl]propane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis[hydroxyethyl]amino]glycine; ER, endoplasmic reticulum; CCT, chaperon containing tailless complex polypeptide 1 (TCP-1).
The pleiotropic effects of the CFTR mutation indicate that the CFTR deficiency reverberates on various cellular processes and therefore probably on the expression, functions, and interactions of the numerous proteins underlying these processes. CFTR interacts with various other proteins such as transport proteins, membrane receptors, proteins of routing and degradation machinery, and proteins of cytoskeleton through interaction with PDZ domain-containing proteins. These interacting proteins are supposed to form a complex protein network whose assembling modalities participate in the modulation of CFTR function (3, 4).

On the other hand, a growing body of evidence indicates that the CF phenotype depends on modifier factors (5, 6). It has been observed that genes such as mannose-binding lectin, glutathione S-transferase, transforming growth factor-β1, tumor necrosis factor α, β₂-adrenergic receptor, HLA class II antigens, and ClCA are linked to the pathogenesis of CF. Whether some of them are part of the CFTR protein network is presently unknown.

Most of the proteins directly involved in the CFTR complexes or indirectly dependent on CFTR function remain to be identified. In this perspective, it appeared likely that proteomics could help us to get a more complete and comprehensive picture of the effect of CFTR mutations by allowing the characterization of changes in protein expression, interactions, and functions that are induced by the CFTR defect.

This approach was successfully used in our laboratory (7) by applying conventional 2D electrophoresis on the total protein fraction of colonic crypts isolated from cftr<sup>−/−</sup> mice. However, this approach is not optimal for the investigation of membrane proteins. For example, ion channels, which are the major players in the defective transepithelial ion transport in CF, are difficult to resolve by conventional 2D gel analysis due to their relatively low abundance compared with soluble proteins, their hydrophobicity, and their prevalent alkaline nature, which seriously compromise their resolution by electrofocusing (8, 9).

Several solutions have been developed to overcome the technical difficulties represented by the analysis of membrane proteins. For example, fractionation of cell lysates and biochemical enrichment has been reported to reduce pattern complexity on 2D gels and thus to improve visualization of low abundance proteins. Alternatively isoelectric focusing can be eliminated (one-dimensional electrophoresis), modified, or judiciously replaced with a different separation technique such as blue native (BN)-PAGE.

BN-PAGE, which appears today as a very promising solution for the investigation of membrane proteomes (10), was initially developed by Schägger and von Jagow (11) to separate intact and functional mitochondrial membrane protein complexes responsible for oxidative phosphorylation. This technique offers the unique advantage of separating native protein complexes present in membrane protein samples without dissociating them. It consists of polyacrylamide gel electrophoresis, where the non-denaturing compound Coomassie Blue G250 is added to the sample and to the electrophoresis buffers, to confer a negative charge on the protein complexes so they can migrate intact toward the anode.

Combining BN-PAGE with SDS-PAGE was shown to result in the separation of several individual subunits of the resolved complexes, offering an interesting two-dimensional electrophoresis approach that allows the profiling of membrane proteins and the characterization of their associations within the membranes. This alternative 2D method was successfully applied to screen for oxidative phosphorylation complex components in mitochondrial encephalomyopathies (12), Parkinson disease (13), and Alzheimer disease (14) as well as for the analysis of cytochrome c oxidase deficiency, indicating that this approach is applicable to clinical studies. Until now, the use of BN/SDS-PAGE for membrane proteome analysis in mammalian cells was only limited to purified mitochondria membrane fractions except for the analysis of raft domains (15), for endoplasmic reticulum (16), and for the very recent study of the microsomal membrane fraction from platelets (17). Here we used this technique for the study of the total membrane proteome of colonic crypt tissue from a knock-out CF mouse model (cftr<sup>−/−</sup>, cftr<sup>tm1Linc</sup>) that shows an intestinal phenotype very similar to that of the human intestinal disease (18). Indeed these mice present runting and failure to thrive, goblet cell hyperplasia, crypt dilatation, and intestinal obstruction (bearing similarity to meconium ileus, which is present in 10–15% of CF patients (19)) with resulting perforation, peritonitis, and death.

In the present study, 2D BN/SDS-PAGE revealed impaired expression of complexes containing the mClCA3 protein in the colon of the cftr<sup>−/−</sup> mice and proved to be an efficient tool for investigation of the membrane proteome from limited amounts of crude membrane preparation. On the basis of this finding, we investigated mClCA3 expression in normal (cftr<sup>+/+</sup>) and cftr<sup>−/−</sup> mice by complementary expression analyses such as immunoblotting and immunohistochemistry. The data were supplemented by an ex vivo functional assay intended to investigate the mucin secretory response related to a known mClCA3 function.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Animals—**All reagents necessary for sample preparation, electrophoresis, and mass spectrometry were of highest grade available. All chemicals were from Sigma except when mentioned in the text.

Mice used for this study were 3–4-week-old C57BL/6 males bred and genotyped in the Animal Core Facility at the Centre de Distribution, Typage and Archivage CNRS, Orléans, France. Mice lacking CFTR expression (cftr<sup>−/−</sup>) were of cftr<sup>tm1Linc</sup> genotype (20). All mice were allowed food and water ad libitum until the time of death.

**Crypt Isolation and Sample Preparation—**Animals were killed by cervical dislocation, and their distal colon was removed and immediately rinsed with cold HEPES-buffered solution (10 mM HEPES, pH 7.2, 140 mM NaCl, 47 mM KCl, 1 mM MgCl₂). Crypts were isolated according to the method described by Krietsmeier et al. (21). Briefly
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colons were opened longitudinally and immersed in Ca\textsuperscript{2+}-free solution (0.2 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.8 mM Na\textsubscript{2}HPO\textsubscript{4}, 107 mM NaCl, 45 mM KCl, 10 mM glucose, 10 mM EDTA, protease inhibitor mixture (Complete protease inhibitor mixture, Roche Applied Science), pH 7.2). The crypts were then separated from connective tissue and muscle layers by vigorous vortexing. Isolated crypts were collected by a brief centrifugation (1000 \times g, 1 min) and strongly homogenized using a tight fitting glass homogenizer in a hypotonic lysis buffer (20 mM Tris, pH 7.4, 25 mM NaCl, 25 mM MgCl\textsubscript{2}, 1 mM EDTA, protease and phosphatase inhibitors (phosphatase inhibitor mixture II from Sigma)). The homogenate was then centrifuged at 3500 \times g for 10 min to eliminate cell debris, nuclei, and the largest mitochondria. The supernatant was kept on ice while the pellet was resuspended, homogenized and centrifuged again. The combined supernatants were centrifuged at 120,000 \times g for 1 h at 4 \degree C, and the final pellet, containing crude membranes, was kept frozen at -80 \degree C until use, generally within the next 24 h.

To solubilize membrane proteins and complexes, the membrane pellet was vigorously pipetted in 40 \mu l of extraction buffer containing 750 mM aminocaproic acid, 50 mM BisTris/HCl, pH 7.0, at 4 \degree C. Four microliters of 12.5\% Triton X-100 were then added to the suspension. After incubation on ice for 20 min with vortexing every 5 min, insoluble membrane material was pelleted at 120,000 \times g for 15 min. The protein concentration of the samples containing solubilized membrane proteins was determined using the Bio-Rad DC protein assay (Bio-Rad). To 40 \mu l of sample, 4 \mu l of 5\% Coomassie Brilliant Blue G250 diluted in the extraction buffer were added. Samples were then briefly centrifuged at 10,000 \times g to eliminate any precipitate and kept on ice until loading on BN gel.

Electrophoresis—BN-PAGE was performed according to a modification of the protocol by Schägger and von Jagow (11). All buffers were adjusted to pH 7.0 at 4 \degree C and filtered through 0.2-\mu m filters.

A 4–10\% gradient gel with a 3.5\% stacker was poured in the Bio-Rad Mini Protean III Cell using 1-mm spacers. Rhinohide\textsuperscript{TM} (Molecular Probes Europe, Leiden, Holland) was added to the acrylamide solution to ensure rigidity of the gel and thereby to limit distortions that could further affect reproducibility of the protein patterns.

The cathode buffer (15 mM BisTris/HCl, 50 mM Tricine) containing 0.02\% (w/v) Coomassie Brilliant Blue G250 and the anode buffer (50 mM BisTris/HCl) were chilled to 4 \degree C before samples (150 or 250 \mu g of protein) were loaded. Forty micrograms of a ferritin solution diluted in 25 mM ammonium bicarbonate were loaded. Forty micrograms of a ferritin solution diluted in 25 mM ammonium bicarbonate, 0.5% (w/v) SDS, and 15 mM \beta-mercaptoethanol were added to the anode buffer (50 mM BisTris/HCl) for 30 min on ice. The cathode buffer was replaced by the same buffer containing 0.005\% of the dye, and the electrophoresis was continued overnight at 8 mA with voltage set at 120 V. After the dye front had run off the gel, the lanes were cut out and washed with dissociating solution (1\% (w/v) SDS, 1\% \beta-mercaptoethanol) for 10 min at room temperature. During the first electrophoresis, a second dimension 10\% Tricine-SDS gel with a 4\% upper gel was poured in Hoeffer plates (1.5-mm spacers). The excised lanes were then inserted between the glass plate assembly and sealed with hot agarose solution comprising 0.7\% (w/v) agarose, 0.5\% (w/v) SDS, and 15 mM \beta-mercaptoethanol. Two gel strips were placed adjacent on a single Tricine gel to facilitate further comparison of protein profiles. They were separated by a 5-mm well reserved for the molecular weight standard. A dissociating solution (1\% SDS, 150 mM \beta-mercaptoethanol) was added on top of the sealed strips and allowed to diffuse into the gel for 10 min. Gel plates were then placed in the electrophoretic tank and overlaid with 500 \mu l of 2\% Laemmli buffer with 5\% \beta-mercaptoethanol. Electrophoresis was performed at 20 mA for 1 h and then limited to 50 mA and 200 V for 6 h. Molecular weight standards (Bio-Rad) were loaded when the dye front reached the level of the bottom of the only well.

Silver Staining and Image Analysis—Proteins were visualized using the silver staining method performed according to Shevchenko et al. (22). Briefly gels were fixed in 50\% methanol and 10\% acetic acid followed by washing three times for 20 min in milli-Q water. Gels were then sensitized by incubating in 0.02\% sodium thiosulfate followed by washing for 20 s in milli-Q water. Gels were immersed in 0.2\% silver nitrate for 45 min and then rinsed twice for 20 s in milli-Q water. The development stage was carried out in 2\% sodium carbonate and 0.05\% formaldehyde (37\%). Finally the reaction was terminated with 40\%/liter Tris base and 2\% acetic acid.

Analysis of the 2D gel image was carried out using ImageMaster 2D Elite software, version 4.01 (Amersham Biosciences). Three separate double gels with similar staining intensity were analyzed.

In-gel Digestions—Individual protein spots were manually excised and recovered in Eppendorf tubes containing 1\% (w/v) acetic acid solution.

Argentic salts were removed from the spots by incubation in an equal mixture of A and B solutions from the Invitrogen silver staining kit, washed for 10 min with milli-Q water and 10 min with 12.5 mM ammonium bicarbonate in 50\% acetonitrile, and then dehydrated in 100\% acetonitrile.

For cysteine reduction and alkylation, dried gel pieces were incubated at 56 \degree C for 45 min in a solution containing 10 mM DTT and 25 mM ammonium bicarbonate. This solution was removed and replaced by 55 mM iodoacetamide in 25 mM ammonium bicarbonate. Following incubation for 45 min in the dark, gel pieces were washed with 25 mM ammonium bicarbonate to remove excess reagents and subsequently dehydrated with 100\% acetonitrile. Dried gel pieces were rehydrated with a trypsin solution (trypsin (Promega, Charbonnieres, France) in 25 mM ammonium bicarbonate) for 30 min on ice. The remaining trypsin solution was discarded to limit trypsin autoalyysis. Gel pieces were then overlaid with 25 mM ammonium bicarbonate and incubated overnight at 37 \degree C. After digestion, the supernatant containing the trypptic peptides was recovered. The remaining peptides were sequentially extracted from the pieces of gel by two successive 10-min sonications in 12.5 mM ammonium bicarbonate, 50\% acetonitrile, 0.1\% TFA, and pure acetonitrile. The supernatants were pooled, dried in a SpeedVac concentrator, and then reconstituted in 5 \mu l of TB buffer (50\% acetonitrile, 0.1\%TFA).

Mass Spectrometry Analysis and Database Searching—Peptides were analyzed by MALDI-TOF MS using an Autoflex instrument (Bruker Daltonics). Samples (0.5 \mu l) were spotted onto a steel target plate (Bruker Daltonics) together with a 1:3 dilution of a saturated \alpha-cyano-4-hydroxycinnamic acid solution in TB buffer. The spots were allowed to air dry for homogenous crystallization.

The instrument was operated in positive ion reflector mode. Each spectrum was the cumulative average of 250–450 laser shots. Mass spectra were first calibrated in the closed external mode using the peptide mixture standard Peptide Mixture-1 from Bruker Daltonics, sometimes using the internal statistical mode to achieve maximum calibration mass accuracy, and analyzed with FlexAnalysis software, version 2.0 (Bruker Daltonics). Peptide mass peaks from each spectrum were submitted to the Mascot peptide mass fingerprint search form (www.matrixscience.com) for analysis with BioTools software, version 2.1 (Bruker Daltonics).

The search included peaks with a signal-to-noise ratio greater than 4. The peak list for each sample was sent into and used to query the non-redundant Mass Spectrometry Protein Sequence Database (MSDB) for protein identification. Standard settings included the following: enzyme, trypsin; missed cleavages, one; fixed modifications, none selected; variable modifications, oxidized methionine and carbamidomethylated cysteine; protein mass, blank; mass values, MH\textsuperscript{+} (monoisotopic); mass tolerance, varied between 75 and 100 ppm depending on the sample.

Immunoblot Analysis—Crypts of distal colon from control and cftr\textsuperscript{−/−} mice were lysed using the method described above except
that 3500 and 120,000 $\times g$ pellets containing insoluble cell material were pooled.

For lung extracts, animals were killed by cervical dislocation, and lungs were removed and immediately rinsed with cold HEPES-buffered solution (10 mM HEPES, pH 7.2, 140 mM NaCl, 47 mM KCl, 1 mM MgCl$_2$). Lungs were strongly homogenized using a tight fitting glass homogenizer in a hypotonic lysis buffer (20 mM Tris, pH7.4, 25 mM NaCl, 2.5 mM MgCl$_2$, 1 mM EDTA, protease and phosphatase inhibitors). Unlysed cells and nuclei were removed from the cell homogeneous by centrifugation (900 $\times g$ for 10 min at 4 °C). The homogenate was then centrifuged first at 3500 $\times g$ for 20 min to eliminate the largest mitochondria and then at 120,000 $\times g$ to pellet the remaining membranes.

Membrane proteins were extracted from crude membrane preparations using an SDS detergent lysis buffer (2% SDS, 10 mM Tris, pH 6.8, protease inhibitor mixture). After vigorous vortexing at room temperature and three 1-min sonications, the lysates were clarified at 20,000 $\times g$ at 15 °C for 20 min. Protein concentration was determined using the Bio-Rad DC protein assay.

Total membrane protein extracts from crypts of the distal colon were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad) according to the standard protocol. Membranes were probed with an anti-mClCA3 antibody, $\alpha$-p3a (1:1000) or $\alpha$-p3b (1:2000) (23).

Densitometric analysis of immunoblots was carried out using the ImageMaster 2D Elite software, version 4.01 (Amersham Biosciences). Band intensity from the CF samples was normalized to the value obtained for the wild type sample of each experiment (which was given an arbitrary value of 100). The calculated values are semi-quantitative and are only meant to give some relative information on band intensities.

Immunohistochemistry—Mouse intestinal distal colon was rapidly rinsed in PBS, recovered with Bright Cryo-M-Bed (myNeurolab, St. Louis, MO), and immediately frozen in liquid nitrogen. Lungs were first flushed with PBS containing 50% Bright Cryo-M-Bed, recovered with 100% Bright Cryo-M-Bed, and frozen in liquid nitrogen. Six-micrometre cryosections of mouse tissues were fixed with cold acetone for 10 min at 4 °C. The immunohistochemical procedures were performed as described elsewhere (24). Briefly all steps were carried out in a humid chamber. Mouse sections were rehydrated in PBS, pH 7.4, and permeabilized with 0.25% Triton X-100 in PBS. Nonspecific binding sites were blocked with 10% FCS and 3% BSA for 1 h at room temperature. Sections were incubated with the primary anti-mClCA3 antibody 3b in 10% FCS and 1% BSA overnight at 4 °C (working dilution for the rabbit anti-mClCA3 antibody 3b was 1:8000). mClCA3 was visualized with Alexa 488-conjugated goat anti-rabbit IgG (heavy + light) secondary antibody (Molecular Probes) diluted 1:1000. The nuclei were visualized with propidium iodide contained in the Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Tissue sections were examined under a Leica confocal laser scanning microscope with argon ion lasers appropriate for Alexa 488 and propidium iodide. Images were collected with Leica 10 $\times$ or 40 $\times$ oil objectives.

Mucus Secretion Assay—Distal colons were harvested from 3-week-old male mice. For each experiment, the colon was cut into two equal parts that were opened longitudinally and preincubated for 10 min in an oxygenated Ringer’s solution maintained at 37 °C and containing 100 $\mu$M niflumic acid or DMSO (1:1000 dilution). The composition of the Ringer’s solution was 145 mM NaCl, 1 mM MgCl$_2$, 3 mM KCl, 2 mM CaCl$_2$, 1 mM HEPES, pH 7.4. Samples were pinned mucosal side up on a wet 8.0-$\mu$m Millipore filter and mounted between the two cells of home-made microchambers maintained at 37 °C. The apparatus, based on the chamber technology used in electrophysiological studies (Ussing chambers), allowed the perfusion of different solutions at the basal side. A constant powerful oxygen bubble flush on the mucosal surface was maintained during the whole experiment to oxygenate the tissue and to strongly homogenize the mucosal chamber content. The upper, mucosal, chamber was filled with 95 $\mu$l of Ringer’s solution. Aliquots (10 $\mu$l) were periodically replaced by the same volume of Ringer’s solution for glycoprotein measurements. Samples were mixed with 2 $\times$ Laemmli sample buffer and loaded on 11% SDS-polyacrylamide gels. Proteins were allowed to pass through the 3.75% stacking gel, and their migration was stopped when the dye front penetrated 5 mm into the 11% resolving gel. This allowed concentration of all the proteins into a sharp band.

Gels were fixed in 5% acetic acid and 50% methanol, and glycoproteins were first oxidized by a periodic acid treatment and then stained using the 300 mM excitable fluorescent hydrazide Pro-Q Emerald dye (Molecular Probes). The Pro-Q Emerald glycoprotein stain reacts with periodate-oxidized carbohydrate groups, generating a bright fluorescent signal localized to glycoproteins when gels are placed on a UV transilluminator. Detection sensitivity and linear dynamic range of the dye have been meticulously evaluated by Steinberg et al. (25) for various model glycoproteins visualized on polyacrylamide gels. Most of the glycoproteins tested by Steinberg et al. (25) were readily quantified over a 500–1000-fold linear range except for two of them that showed a linear range of 125-fold. We used the stain as indicated by the supplier using a UV transilluminator (Bio-Rad) for visualization of glycoproteins. In our experimental conditions, the limit of detection was 100 ng for the $\alpha$1-acid glycoprotein (Candy Cane standard glycoproteins from Molecular Probes) as well as for type 1S mucins purified from bovine submaxillary glands (Sigma). We verified that the dynamic range was linear within the 100–1250-ng range for the tested glycoproteins. The quantity of glycoproteins detected in samples was estimated to be 150–1000 ng relative to the $\alpha$1-acid glycoprotein standard. After image acquisition, the intensity of visualized bands was semiquantified using the ImageMaster 2D Elite software, version 4.01 (Amersham Biosciences).

RESULTS

**BN/SDS-PAGE Analysis of Membrane Proteins from Murine Colonic Crypts**—The technique of BN-PAGE was initially developed for analysis of the mitochondrial proteome, allowing characterization of many proteins that cannot be resolved using conventional IEF SDS-PAGE. It was sensitive and reproducible enough to be used to screen for mitochondrial protein defects in clinical studies and prenatal diagnosis. We chose to use this approach to investigate not only the mitochondrial but the whole membrane proteome of a tissue particularly affected by the CFTR defect in the mouse model, the colonic epithelium.

Crypts (Fig. 1A) were isolated from the distal colon of cftr$^{+/+}$ and cftr$^{-/-}$ mice to eliminate nonrelevant tissues such as muscular and connective tissue that could compromise the quality and significance of further 2D analysis. Crude membranes were prepared from this material by a classical cell fractionation centrifugation method to selectively enrich protein from membranes and organelles other than nucleus (e.g. mitochondria, ER, and Golgi apparatus) and thereby reduce spot pattern complexity.

Fig. 1B shows representative partial proteomic maps of membrane proteins (150 $\mu$g) solubilized from colonic crypts from a wild type (left side) and a cftr$^{-/-}$ (right side) mouse.
separated by 2D BN/SDS-PAGE gels. The proteins from cftr<sup>+/+</sup> and cftr<sup>-/-</sup> mice were loaded onto the same Tricine-SDS 2D gel to minimize the effects of intergel variability and facilitate comparison with further protein patterns. Most of the 50–60 individual protein spots routinely detected in these conditions by silver nitrate staining were excised and subsequently submitted to trypsin digestion and mass spectrometry analysis to be identified. Table I lists the identified protein from the spots.

Under our experimental conditions for BN-PAGE (4–10% acrylamide gradient gels), only complexes above 300 kDa...
were efficiently resolved, leading to the detection of a large number of complexes in the 300–600-kDa range. Most of those that were easily detected and identified correspond to mitochondrial multienzyme complexes responsible for oxidative phosphorylation: the NADH-ubiquinone oxidoreductase complex (complex I), the ubiquinol-cytochrome c reductase complex (complex II), and the ATP synthase complex (complex V). The presence of these well known complexes indicates that in our conditions of sample preparation and electrophoresis, resolution of multiprotein complexes is achieved. One of the largest and most intensely stained spots that appeared on the 2D gels, spot w, has an apparent electrophoretic mobility of 75 kDa in the SDS-PAGE dimension. This spot was unambiguously identified as the mClCA3 protein (Fig. 2), a calcium-activated chloride channel that has been shown to be associated with the mucin granule membrane of gastrointestinal, respiratory, and uterine goblet cells and other mucin-producing cells (23). The corresponding complex migrates at more than 900 kDa in the BN gel. It appears to contain few spots with a weaker intensity compared with that

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**Expression of mCICA3 in CF Mice**

**TABLE I**

Proteins identified from BN/SDS-PAGE maps

| Spot | Accession number | Protein name | Score | Sequence coverage | Molecular mass | TMD | pl | Localization |
|------|------------------|--------------|-------|-------------------|----------------|-----|----|-------------|
| a    | BAB28666         | Ubiquinol-cytochrome c reductase complex core protein 1 | 169   | 49.6              | 50             | 52.768 | 1   | 5.75        | Mitochondrial inner membrane |
| b    | Q8BK11           | Ubiquinol-cytochrome c reductase complex core protein 2 | 95    | 45.7              | 46             | 48.262 | 1   | 9.33        | Mitochondrial inner membrane |
| c    | Q9DOM3           | Cytochrome c, heme protein | 121   | 41.6              | 34             | 35.327 | 2-3 | 9.24        | Mitochondrial inner membrane intermembrane space, ER, Golgi membrane form a stable homohexameric structure |
| d    | Q8CEG4           | Valosin-containing protein (VCP) (p97) | 200   | 37.3              | 98             | 97.0   | 0   | 89.364      | ER, Golgi membrane form a stable homohexameric structure |
| e    | Q8K0P6           | Similar to human FCγ-binding protein | 67    | 13.8              | 116            | 127.15 | 2-3 | 4.98        | Plasma membrane |
| f, w, v, x | JG0168 | Gob-5 | 414   | 49.7              | 75             | 100.071 | 2   | 5.67        | Secretory granule |
| g    | CAA43675         | Na+/K+-exchanging ATPase β chain | 69    | 34.2              | 62             | 31.482 | 1   | 8.32        | Plasma membrane |
| h    | Q91Z09           | Similar to ATPase, Na+/K+ transporting α1a.1 polypeptide | 207   | 31                | 98             | 112.982 | 9   | 5.3         | Plasma membrane |
| i    | J1C4731          | H+-transporting two-sector ATP synthase α chain | 211   | 50.5              | 56             | 59.752 | 0   | 9.22        | Mitochondrial inner membrane |
| j    | BAB26846         | ATP synthase β chain | 201   | 41.2              | 54             | 56.26  | 1   | 5.19        | Mitochondrial inner membrane |
| k    | Q9D9DT10105      | ATP synthase, H+-transporting, mitochondrial F1 complex, γ subunit | 70    | 27                | 32             | 30.237 | 1   | 9.43        | Mitochondrial inner membrane |
| l    | BAB22481         | ATP synthase, H+-transporting F0 complex subunit b, isoform 1 | 80    | 31.6              | 25             | 28.930 | 2-3 | 9.11        | Mitochondrial inner membrane |
| m    | BAB28667         | ATP synthase e chain | 66    | 75.7              | 8              | 8.1    | 9.34 | Mitochondrial inner membrane |
| n    | BAC29641         | NADH-ubiquinone oxidoreductase 75-kDa subunit | 71    | 13.9              | 79             | 79.749 | 0   | 5.51        | Mitochondrial inner membrane |
| o    | Q91Z1234         | Propionyl-CoA-carboxylase α subunit | 92    | 22.2              | 76             | 79.660 | 0   | 7.02        | Mitochondrial |
| p    | BAC37293         | NADH-ubiquinone oxidoreductase 49-kDa subunit | 66    | 24.2              | 49             | 52.625 | 1   | 6.52        | Mitochondrial inner membrane matrix side |
| q    | AAH05780         | NADH-ubiquinone oxidoreductase 39-kDa subunit | 68    | 25.6              | 37             | 42.509 | 3   | 9.75        | Mitochondrial matrix |
| r    | S43062           | T-complex protein 1, γ subunit (TCP-1-γ) (CCT-γ) (matrinin) | 79    | 28.3              | 64             | 60.630 | 1   | 6.28        | Cytoplasmic complex, known as chaperonin TRIC or CCT, of about 850–900 kDa |
| s    | Q8CS06           | CCT β chain chaperonin subunit 8 (i) | 70    | 29                | 63             | 49.886 | 1-2 | 5.55        | Cytoplasmic |
| t    | S43058           | T-complex protein 1, ε subunit (TCP-1-ε) (CCT-ε) | 68    | 26.8              | 60             | 59.652 | 0   | 7.95        | Cytoplasmic |
| u    | TCPB MOUSE       | T-complex protein 1, β subunit (TCP-1-β) (A45) | 95    | 29.5              | 60             | 58.066 | 1-2 | 8.24        | Cytoplasmic |
| v    | AAH05780         | NADH-ubiquinone oxidoreductase 39-kDa subunit | 68    | 25.6              | 37             | 42.509 | 3   | 9.75        | Mitochondrial matrix |

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*a* The letters correspond to the marked letters on the master gel in Fig.1. MS analysis of the spot w is indicated in the table by an asterisk (*).

*b* MSDB accession number.

*c* Mowse score from Mascot search at www.matrixscience.com. The score at which statistical significance (p < 0.05) occurred was 61.

*d* Molecular mass as defined by ExPASy software at www.expasy.org.

*e* Number of transmembrane regions (TMD) predicted by TMsearch software at www.ch.embnet.org. The most probable number of transmembrane regions is in bold.

*f* pI value of proteins as defined by ExPASy software at www.expasy.org.

*g* Predicted at 89.364 but observed at 97 kDa (38, 39).
of mClCA3, suggesting that the 75-kDa mClCA3 protein is the main component of this large complex. Two other spots (i and j, Table I) were also differentially expressed between cftr+/−/+ and cftr−−− mice, but they were not further analyzed in the present study. The differences in other spots (Fig. 1B, marked with *) were not reproducible.

In the cftr+/−+ colon, after a long silver staining procedure, the 75-kDa mClCA3 spot has a comet-like appearance, extending to lower molecular weights on BN-PAGE and to higher molecular weights on SDS-PAGE. The former may be the result of difficulties in the migration of large complexes on BN-PAGE, whereas the latter suggests that larger forms of the

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**Fig. 2. Spot w identification by MALDI-TOF MS analysis.** The w protein band was excised from the 2D gel and subjected to in-gel digestion followed by MALDI-TOF MS analysis. A, a typical mass spectrum acquired during a MALDI-TOF analysis from a trypsin digest of spot w. Spectra were processed using FlexAnalysis software, and peptide peaks were submitted to the Mascot peptide mass fingerprint search engine (www.matrixscience.com) through the Bioworks software (Bruker Daltonics). The annotated peaks correspond to those peptides that match with the mClCA3 protein. B, Mowse score is −10(log(p)), where p is the probability that the observed match is a random event (53). In this analysis, scores >61 indicate identity or at least extensive homology (p < 0.05). A probability-based Mowse score of 414 indicated that the identified peptides match the mClCA3 protein (referenced as gob-5 protein) sequence with a high degree of certainty. On the histogram of the score distribution, the shaded area represents the scores with the greatest statistical uncertainty. The results showed that matching of the spot w tryptic peptides with other protein sequences was unsuccessful. C, the Mascot search showed that the matched peptides (in bold) from the isolated w protein accounted for 49.7% of the protein sequence of mClCA3.
mClCA3 protein are present in the complex (Fig. 1B, left panel). Two spots of 110 and 48 kDa (spots v and x) located in the vertical line on SDS-PAGE and corresponding to the >900-kDa mClCA3-containing complex were identified as identical to the mClCA3 protein with a significant score (277 and 64, respectively). These different species of the mClCA3 protein correspond in size to the mature glycosylated and truncated colon-specific forms described previously by Leverkoehne and Gruber (23).

Comparison of Protein Maps Obtained from cftr+/+ and cftr−/− Mice—Comparison of the membrane protein profiles from cftr+/+ and cftr−/− colonic crypts revealed that the mClCA3-containing complex was relatively under-represented in the cftr−/− colon as confirmed by a densitometric analysis of the mClCA3 bands (Fig. 1C). Moreover no or poor signal corresponding to the comet products of the SDS-PAGE mentioned above was detected above the 75-kDa mClCA3 spot, suggesting that higher mClCA3-related forms are also under-represented in the sample from cftr−/− mice.

Differential Expression of mClCA3 Protein in Colon and Lung of cftr−/− Mice—The comparison of the mClCA3 expression pattern detected on BN/SDS-PAGE gels suggested that overall expression of the mClCA3 protein may be impaired in the colonic crypts of cftr−/− mice. To test this hypothesis, we performed an immunoblotting analysis of the membrane fraction isolated from wild type and CF crypts using specific mClCA3 antibodies. As shown in Fig. 3, mClCA3 antibodies detected a major protein of 75 kDa and one minor band of 110 kDa in crypt extracts from wild type animals (seen only in the middle and right panels of Fig. 3). In
comparison, levels of mClCA3 bands in the corresponding cftr−/− tissue were significantly lower (normalized to galec-tin-4, a well known membrane-associated protein highly ex-
pressed in the colon).

Because lung tissue expresses CFTR and mClCA3 proteins (23) and is particularly relevant to CF, we next investigated mClCA3 expression in lung of wild type and cftr−/− mice.

Immunoblot analysis of membrane extracts from lung revealed that mClCA3 is slightly decreased in the lung of the cftr−/− mice (Fig. 3, bottom). All the labeled cells exhibited a granular staining pattern throughout the cytosol. A faint staining of the mucous layer lining the intestinal surface and the luminal surface of the crypts was also detected. B, distribution pattern of the mClCA3 protein (green) in lungs of CF (c, d) and control (a, b) mice. mClCA3 protein was detected in bronchioles and in the alveolar region. All labeled cells exhibited a granular staining pattern throughout the cytosol. Most of the epithelial cells lining the bronchioles showed an intense caplike staining pattern at the luminal surface. The pattern looked similar in the two mice types, but CF mice showed a weaker labeling intensity.

Expression of mClCA3 in CF Mice

Fig. 4. Cellular distribution pattern of the mClCA3 protein in colon and lung of CF and wild type mice as detected by immunohis-
tochemistry using acetone-fixed cryosections. Tissue sections from cftr−/− and wild type mice were incubated in parallel with α-p3b (1:8000) and with an Alexa 488-conjugated goat anti-rabbit IgG (1:1000) secondary antibody. Nuclei are stained with propidium iodide (red). A, distribution pattern of the mClCA3 protein (green) in colon of cftr−/− (a, b, d, and f) and cftr−/− (c, e, and g) mice. The mClCA3 protein was mainly detected in the upper two-thirds of the crypts of control mice (a and b), whereas weak or no staining was observed in CF mice (c and g). All the labeled cells exhibited a granular staining pattern throughout the cytosol. A faint staining of the mucous layer lining the intestinal surface and the luminal surface of the crypts was also detected. B, distribution pattern of the mClCA3 protein (green) in lungs of CF (c, d) and control (a, b) mice. mClCA3 protein was detected in bronchioles and in the alveolar region. All labeled cells exhibited a granular staining pattern throughout the cytosol. Most of the epithelial cells lining the bronchioles showed an intense caplike staining pattern at the luminal surface. The pattern looked similar in the two mice types, but CF mice showed a weaker labeling intensity.
that mClCA3 expression is reduced in cftr−/− mice and raised the possibility that mClCA3-dependent functions could be defective in the cftr−/− colon.

Comparison of Carbachol-induced and Niflumic Acid-sensitive Glycoprotein Secretion in the Distal Colon of cftr+/+ and cftr−/− Mice—A growing body of evidence suggests that mClCA3 and its human counterpart hClCA1, which are two members of a family of calcium-activated chloride channels (CaCCs), play a crucial role in mucus production by goblet cells (26, 27). The most common blocker for native CaCCs is niflumic acid (NA), a non-steroidal anti-inflammatory drug classically used to identify anion currents as CaCCs in different tissues. NA is regarded as a potent and reversible blocker of calcium-activated chloride conductances (28–31). Furthermore, recent data suggest that it also reduces mucus secretion by acting on mClCA3 and hClCA1 proteins (32, 33). Therefore, the ability of NA to inhibit both chloride and mucus secretion driven by mClCA3 makes it a good candidate for probing the involvement of these proteins in secretion processes within mouse epithelia.

To determine whether CaCC-related mucus secretion, i.e. possibly mediated by mClCA3, is affected in the cftr−/− colon, the response of distal colon to the mucin secretagogue carbachol in the presence or absence of NA was investigated in an ex vivo assay (Fig. 5). In these experiments, pieces of distal colon were mounted in perfusion chambers, and mucin secretion was measured as accumulation of glycoproteins on the mucosal side as detected by Pro-Q Emerald reagent (see “Experimental Procedures” for details). Several lines of evidence confirm that the cholinergic agonist carbachol induces Ca2+-dependent chloride secretion as well as the degranulation of goblet cells in various epithelia (5, 34, 35) and in particular in colonic crypts (36). Consistent with these data, the results presented in Fig. 5 show that carbachol (20 μM) efficiently stimulated glycoprotein release by the wild type distal colon epithelium. This response to the secretagogue
was a rapid phenomenon, reaching its maximum after 4–6 min of stimulation under our experimental conditions. Application of NA (100 μM), but not of an inhibitor of CFTR (CFTR-inh172, 10 μM) (37), markedly diminished the carbachol-stimulated glycoprotein release suggesting that CaCC, but not CFTR, participates in this secretion process. Distal colon explants from cftr−/− mice did not respond to carbachol, their glycoprotein release in the presence of the secretagogue being close to the secretion levels observed in unstimulated colons from cftr+/+ mice. Taken together, these results showed that a carbachol-induced NA-inhibited glycoprotein secretion is impaired in the distal colon of cftr−/− mice and supported the conclusion that CICA function may be defective in this tissue.

**DISCUSSION**

**Membrane Preparation and BN/SDS-PAGE Analysis**—In an attempt to investigate the membrane proteome of colonic crypts harvested from individual mice, we used a rudimentary differential centrifugation method to fractionate cell lysates followed by a BN/SDS-PAGE analysis. The basic enrichment procedure was intended to reduce the complexity of protein patterns and allow recovery of enough membrane proteins from plasma membrane and organelles to be above the threshold of detection on 2D gels. Under these conditions, half of the proteins (54%) that were identified from the BN/SDS gels were components of mitochondrial complexes (Table I). This result was expected because we used a high salt solubilization buffer very similar to those described by Schägger and von Jagow (11) in the original method designed for mitochondrial complexes. However, the sample preparation also provided many non-mitochondrial membrane proteins: Na+/K+-ATPase, known to be located in the basolateral plasma membrane; mCICA3, which was shown to be associated with secretion vesicles (23); and valosin-containing protein, described as an homohexamer, which has several differ-
ent functions at the level of ER and Golgi compartments (38–40), although it has not been referenced as a transmembrane protein. Proteins that are not routinely considered to be related to membranes also appear on the maps. Proteins corresponding to spots r, s, t, and u (Table I, CTT-γ, CTT-θ, CTT-η, CTT-δ, and CTT-β) were described as forming the cytosolic chaperonin CCT (chaperonin containing tailess complex polypeptide 1 (TCP-1)). This macromolecular complex was described to form a 850–900-kDa ring-shaped structure with a large central cavity (41) that assists in the folding of actin, tubulin, and other cytosolic proteins. The association with cytoskeletal components could explain the presence of CCT in a pellet obtained by centrifugation at 120,000 × g. More likely, CCT could be associated with intracellular vesicles as it was originally identified as the chromobindin A complex (42), a multiprotein complex attached to secretory vesicle membranes.

Altogether we show that BN/SDS-PAGE allows effective separation of protein species and complexes from various subcellular origins, including mitochondria, plasma membrane, and intracellular vesicles, present in a typical crude membrane preparation obtained by differential centrifugation prefraccionation. The limited heterogeneity of such an extract did not represent a limiting factor for the correct mapping of membrane proteins using this alternative 2D technique.

**Biochemical Characteristics of Proteins Resolved by BN-PAGE**—Although in-gel tryptic cleavage is disadvantaged in the analysis of hydrophobic regions of proteins, compromising transmembrane protein identification by subsequent MALDI-TOF MS, the results presented in Table I show that 68.2% of the proteins that were successfully identified by MALDI-TOF MS had one to nine putative transmembrane domains, and 31.8% had a pi value higher than 9, probably rendering their separation by the usual 2D gel separation method difficult or impossible. Thus, it can be asserted that the 2D BN/SDS-PAGE technique seems particularly well suited for separation of transmembrane proteins and proteins with high pi values that usually fail to enter the first dimension of 2D IEF/SDS gels, allowing us to conclude that BN/SDS-PAGE constitutes an interesting complement to conventional 2D electrophoresis. Accordingly mClCA3, one of the most abundant proteins on BN/SDS-PAGE, was not detected on the 2D IEF/SDS analysis performed with the membrane protein preparation that we describe in this study (data not shown).

**mClCA3 Migration Profile in BN/SDS-PAGE**—The mClCA3 protein has an apparent electrophoretic mobility of 75 kDa in the second dimension of BN/SDS-PAGE (Fig. 1) as well as in single SDS-PAGE as shown on immunoblots (Fig. 3). This differs from the molecular weight reported by Leverkoehne and Gruber (23) who found an apparent molecular mass of 90 kDa in immunoblot from total cellular extracts obtained from the whole mouse colon. This difference may be due to the experimental conditions used for electrophoresis. Such an explanation seems unlikely because we have found that mClCA3 migrates as a 75-kDa protein in two different conditions, i.e. 8% Tricine-SDS-PAGE and 10% glycine-SDS-PAGE. Alternatively the difference could be explained by the type of cellular extract. This explanation is plausible because, in our study, the 75-kDa mClCA3 protein was present in membrane samples from crypts of the distal colon and in the same preparation from lung, whereas a total protein extract was used by Leverkoehne and Gruber (23).

The protein sequence deduced from the mClCA3 cDNA corresponds to a 913-amino acid polypeptide with a predicted molecular mass of 100.07 kDa. Accordingly *in vitro* translation of mClCA3 results in a 100-kDa product that increases in size to 110 kDa after glycosylation by microsomal membranes (23). However, like other members of the CICA family (such as its human, porcine, and equine counterparts: hClCA1, pCICA1, and eCICA1), mClCA3 post-translational modifications produce shorter final products in native tissues or when expressed in heterologous systems. In this regard, it was shown that several members of the CICA family have potential proteolytic cleavage sites and might be proteolytically processed to shorter size mature forms (43).

The mClCA3 peptide mass fingerprint obtained after tryptic digestion of mClCA3 samples indicates the lack of C-terminal peptides from the amino acid at position 686. None of the eleven peptides in the 1000–3000-Da range expected from the theoretical tryptic digest (with one mis cleavage allowed) were present on MS spectra. This suggests that a proteolytic removal of this C-terminal region of mClCA3 occurs in the tissue. The calculated molecular weight of the 1–685 region is 75.3 kDa (or 73.3 kDa when, in addition, the N-terminal peptide signal sequence (1–21) is cleaved), which is very close to the molecular weight of the mClCA3 detected by BN SDS-PAGE and immunoblot analysis. However, we cannot exclude that the tryptic peptides in this region are not properly recovered from the gel and/or ionized.

The mClCA3-containing complex migrates at ~1000 kDa in the BN gel. This large mClCA3-containing complex migrated systematically, in eight independent experiments, at the same position in the first dimension, and the mClCA3 protein band appeared as a well delimited vertical comet in the second, a pattern that does not correspond to random aggregation of mClCA3 proteins. Nevertheless we cannot exclude the possibility that mClCA3 proteins aggregate during BN analysis to form the high molecular weight complex even though the charged blue dye, Coomassie Brilliant Blue G-250, added in sample and cathode buffers (0.5% and 0.02%, respectively) binds to the hydrophobic protein surface and reduces aggregation. Alternatively mClCA3 could be clustered in microdomains in the vesicle membrane.

Two minor forms of the mClCA3 protein, the 48- and 110-kDa forms described by Leverkoehne and Gruber (23), were also detected by us as minor components of the mClCA3-containing complex. Finally other protein spots not annotated...
on Fig. 1 were also detected in the vertical line of the mClCA3 protein in samples from cfr"+/+" mice. Identification of these putative partners of mClCA3 is in progress.

A Link between mClCA3 and CFTR Expressions—We show that mClCA3 expression is significantly reduced in two murine epithelia lacking CFTR, i.e. in the lung and colon of cfr"−/−" mice. The search for the underlying mechanisms that link the expression of mClCA3 and CFTR function was not within the scope of the present study. Nevertheless the data from two groups point to a possible relation between CFTR (a cAMP-dependent Cl⁻ channel) function and the expression of Ca²⁺-dependent Cl⁻ channels. It has been postulated that an as-yet-unidentified Ca²⁺-sensitive Cl⁻ conductance in the cfr"−/−" mouse rescues the cystic fibrosis mouse from significant airway disease (44–46). There was no molecular entity that could have accounted for this effect until mClCA1, the first identified murine member of the CICA gene family, was described (47). Henceforth in consideration of recent studies on its tissue distribution (23), it can be reasonably proposed that mClCA3 represents this Ca²⁺-dependent Cl⁻ conductance.

However, the role of Ca²⁺-dependent Cl⁻ channels is more complex. For example, it is known that the lethal intestinal pathology of CF is associated with the absence of a Ca²⁺-activated pathway for Cl⁻ secretion, whereas expression of a Ca²⁺-sensitive Cl⁻ conductance in the murine intestine is thought to compensate for the lack of CFTR function and to rescue the intestinal phenotype (46, 48). Once again, mClCA3 may constitute this Ca²⁺-dependent Cl⁻ conductance. Consistently Ritzka et al. (6) have shown that the CICA locus is associated with a CFTR-independent, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid-sensitive, residual chloride conductance in the rectal mucosa of CF patients. The authors concluded from genetic and expression studies that hClCA4 may constitute this Ca²⁺-dependent Cl⁻ conductance. Consistently Ritzka et al. (6) have shown that the CICA locus is associated with a CFTR-independent, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid-sensitive, residual chloride conductance in the rectal mucosa of CF patients. The authors concluded from genetic and expression studies that hClCA4, and more likely hCICA1, the human counterpart of mClCA3, could be a modulator of the basic gastrointestinal defect in cystic fibrosis. Hence the reduced expression of mClCA3 protein that we describe in the present study can be thought of as a determinant of the cfr"tm1Unc" mice intestinal pathology.

Impaired Carbachol-induced and Niflumic Acid-sensitive Mucus Secretion in CF Colon—It has been proposed that a Ca²⁺-dependent Cl⁻ channel is implicated in the secretion of mucous glycoproteins (termed mucins) (26, 32, 33). In the present study, we show that a Ca²⁺-stimulated mucin secretion, strongly inhibited by NA, is defective in the distal colon of cfr"−/−" mice.

In the colon, mucin secretion contributes to the barrier function of the epithelium (49). The bulk of mucins originates from the goblet cells located in the epithelium lining the crypts. Regulation of mucus secretion by these cells is not well understood. It involves granule fusion and exocytotic release of granule content at the luminal surface. Most agonists that stimulate mucus secretory response are also potent ion secretagogues, suggesting that both phenomena are linked. Our results suggesting that a CICA-related mucin secretion is defective in cfr"−/−" mice are in agreement with previous observations showing that animals lacking functional CFTR exhibit no Cl⁻ secretory response to agonists that increase intracellular Ca²⁺ (46, 50–52). We propose that CICA and most likely mClCA3, which is localized in goblet cells, are involved in this mucus secretory response. In turn, the under-expression of mClCA3 protein in cfr"−/−" mice participates in the defect of fluid and mucus transport in the colon.

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