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Characterization of Novel Airway Submucosal Gland Cell Models for Cystic Fibrosis Studies

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Key Words

CFTR • Cystic fibrosis • CF cellular model • Epithelial cells • Calu-3 • Submucosal gland • Airway cell line

Abstract

Cultured airway epithelial cells are widely used in cystic fibrosis (CF) research as *in vitro* models that mimic the *in vivo* manifestations of the disease and help to define a specific cellular phenotype. Recently, a number of *in vitro* studies have used an airway adenocarcinoma cell line, Calu-3 that expresses submucosal gland cell features and significant levels of the wild-type CFTR mRNA and protein. We further characterized previously described CF tracheobronchial gland cell lines, CFSMEo- and 6CFSMEo- and determined that these cell lines are compound heterozygotes for the F508del and Q2X mutations, produce vestigial amounts of CFTR mRNA, and do not express detectable CFTR protein. Electrophysiologically, both cell lines are characteristically CF in that they lack cAMP-induced Cl^- currents. In this study the cell lines are evaluated in the context of their role as the CF correlate to the Calu-3 cells. Together these cell systems provide defined culture systems to study the biology and pathology of CF. These airway epithelial cell lines may also be a useful negative protein control for numerous studies involving gene therapy by cDNA complementation or gene targeting.

Introduction

Cystic Fibrosis (CF) is the most common lethal, autosomal recessive disease among Caucasians. It is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, that, primarily functions as a cAMP-activated and phosphorylation-regulated Cl^- membrane channel and as a regulator of various other channels [1-4]. About 1300 sequence variants have been detected in the CFTR gene, most presumed to be disease-causing [5]. However, the predominant mutation is a trinucleotide deletion resulting in the loss of phenylalanine at amino acid 508 (F508del) of the CFTR protein, accounting for approximately 70% of all CF alleles in the Northern European population [2, 6-8].

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CF is characterized by progressive deterioration of lung function (the main cause of morbidity and mortality), pancreatic dysfunction, elevated sweat electrolytes, and male infertility [8, 9]. CFTR protein is localized to the apical membrane of epithelial cells that line the airways, intestine and a variety of exocrine glands [10-14]. However, CFTR is expressed in most native epithelial tissues at very low levels [4, 15], with the exception of the intestine and the airway submucosal glands which appear to have higher endogenous CFTR expression [16-19]. Tracheobronchial submucosal glands secrete liquid essential for the clearance of the macromolecular mucus component of the gland secretory complex from the gland ducts and for maintaining airway surface liquid (ASL) volume to facilitate mucociliary transport [20]. The submucosal glands have also been proposed as the primary site for initiating and sustaining airway disease in CF [21].

Due to the very low levels of endogenous expression, as well as to the limited availability and size of native epithelial tissues, primary cultures and immortalized cell lines constitutively synthesizing the protein have been developed to characterize the biochemical and genetic mechanisms underlying CF [10, 22-28]. A number of immortalized airway epithelial cell lines generated in the past have been critical for enhancing our understanding of the pathways responsible for CF pathology [29-39]. Transformed heterologous cells transfected with wild-type (wt) or mutant CFTR cDNA have also been widely used for biochemical studies [40-44]. Where significant amounts of protein are required, these cell systems have been the models of choice [45]. However, because most of these are non-epithelial and/or are non-polarized epithelial cells or do not normally express CFTR they have a limited applicability for the assessment of vectorial ion transport, secretion, trafficking and other differentiated functions [17, 46].

A number of CF studies have used an lung adenocarcinoma-derived airway submucosal gland cell line, Calu-3 [47] that has been shown to express significant levels of wt-CFTR mRNA and protein [45, 48]. As a complement to these cells we have more fully characterized two previously isolated CF tracheobronchial gland epithelial cell lines, CFSMEo- and 6CFSMEo-. These cell lines are compound heterozygote for the F508del mutation and were originally derived through transformation by an origin of replication defective SV40 containing plasmid, pSVori- [30]. This study further characterizes the cells in terms of the second CFTR mutation, CFTR RNA and protein expression, as well as the cells electrophysiological properties. The use of these cells lines as the CF correlates to Calu-3 cells is discussed, since taken together, they represent ideal cell culture systems to study the biology and pathology of CF.

Materials and Methods

Cells and Cell Transformation

The CFSMEo- (not previously described) and 6CFSMEo- cell lines were generated from freshly isolated submucosal gland epithelial cells from an individual with CF [30, 49]. The cultures of airway epithelial gland cells were transfected with the pSVori- plasmid that contains a replication-deficient simian virus 40 (SV40) genome [37, 50, 51]. Colonies of transformants were isolated or pooled (6CFSMEo- and CFSMEo-, respectively), expanded and partially characterized in terms of their genotype and phenotype. This study elaborates on the original characterization of the 6CFSMEo- cell lines [30] and previously uncharacterized cell line, CFSMEo-.

The cells were grown on transwell inserts or on tissue culture plastic coated with an extracellular matrix cocktail comprised of human fibronectin (FN) (BD Biosciences, Bedford, MA), Vitrogen (V) (Cohesion, Inc., Palo Alto, CA), and bovine serum albumin (BSA) (Biosource/ Biofluids, Camarillo, CA) [22, 52] in DME/F12 (1:1) or MEM supplemented with 10% fetal calf serum (FCS), 1% (v/v) glutamine, 1% pen/strep under 5% CO_2 at 37°C.

Immunocytochemical Staining

Cells were analyzed by immunofluorescence as described previously for the presence the SV40 large tumor antigen (large T-antigen), keratin, and a tight junction specific antigen [37] to verify their epithelial status. The cells were grown on well-slides (Lab-Tek) and stained with the airway epithelial-specific, keratin 18 (K18) anti-cytokeratin antibody, and a monoclonal against the junctional complex adhesion protein, ZO-1. Cytokeratin and tight junctions are hallmarks of epithelial cells and the K18 and ZO-1 antibodies are more specific to airway epithelium and tight junction than were the AE1/AE3 and cell CAM 120/80 antibodies, respectively, used in the an earlier study [30]. All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

Mutation Analysis

The presence of F508del mutation in one of the CFTR alleles had been described previously for both cell lines [30]. To search for the second mutation, genomic DNA from 6CFSMEo- cells was isolated using the Wizard® genomic DNA purification kit (Promega, Madison, WI, USA). All CFTR exons and flanking intron regions (at least 50-bp into each adjacent intron), including the 3' untranslated region (3' UTR), were PCR-amplified using 100 ng of genomic DNA as template in a reaction solution containing (50 µl final volume): 1x PCR buffer I (supplied with AmpliTaq®, Applied Biosystems, Foster City,

Table 1. PCR Primers. *Numbering according to [4]. Legend: PCR primers used for either the analysis of mRNA-derived cDNA or for genotyping. Primers CF1R and CF1-NL were used to identify the Q2X mutation.

PRIMER	Localization (Start/End)*	SEQUENCE
B2R (antisense)	Exon 7 (1022/1041)	5'-GGAAGGCAGCCTATGTGAGA -3' [57]
B2L (sense)	Exon 13 (1901/1920)	5'-AGCCATCAGTTTAC AGACAC-3' [57]
FAM-B3F (antisense) (Fam-labeled)	Exon 8 (1318/1338)	5'-AATGTAACAGCCTTCTGGGAG -3' [58]
C16D (sense)	Exon 10 (1685/1708)	5'-GTTGGCATGCTTTGATGACGCTTC -3' [58]
CF17 (sense)		5'-GAGGGATTTGGGGAATTATTTG -3'
CF7C (antisense)		5'-GAGGGATTTGGGGAATTATTTG -3'
CF8C (antisense)		5'-ATAGGAAACACCAATGATAT -3'
CF1-R (sense)	5' UTR (-134/-115)	5'-CGTAGTGGGTGGAGAAAGC-3'
CF1-NL (antisense)	Intron 1 (185+85/185+66)	5'- CCTTTACCCCAAACCCAACC-3'

CA, USA), 10 pmol of each primer, 200 µM each dNTP, 1.5 U AmpliTaq® DNA polymerase (Applied Biosystems). The primers used and respective PCR conditions, corresponding altogether to 30 different PCR reactions to cover the 27 exons and the 3' trailer, are described elsewhere [53]. Reactions were carried out using an OMN-E thermocycler (Thermo-Hybaid, Woburn, MA, USA) with the "hot lid" on. Specificity of PCR products was confirmed by sizing on agarose gels and stored at 4ºC until analysis by automatic sequencing on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems) using the Big Dye® Terminator v1.1 sequencing kit (Applied Biosystems). The second CFTR mutation in the CFSMEo- was confirmed by PCR and Mwo I restriction fragment polymorphism (RFLP) analysis once it was identified in the 6CFSMEo- cells.

CFTR mRNA Analysis

RNA was extracted from the CFSMEo- and 6CFSMEo- cells grown to confluence on Transwell® filter inserts (Costar, Cambridge, MA, USA) or on FN/V/BSA coated culture dishes with RNeasy® mini kit (Quiagen, Hilden, Germany). The cDNA was generated using random hexamers and Superscript™II RNase H- reverse transcriptase (InVitrogen, Carlsbad, CA, USA), as described previously [54, 55]. CFTR mRNA was DNase-treated and analyzed by standard allele-specific RT-PCR using primers CF17 (exon 9) and CF7C/8C (exon 10; wild-type and F508del mutation, respectively) (Table 1) [56] and or by nested PCR of a primary amplification product from the region encompassing exons 7 to 13 generated with primers B2R and B2L (Table 1) [57]. The nested PCR (second round) was performed in the CFTR region of exons 8 to 10 with the primers Fam-B3F and C16D [58], using 5µl of the reaction solution from the first round of PCR amplification.

Allele-specific PCR amplification was carried out in a 30 µl solution containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.03 U/µl Platinum Taq polymerase (InVitrogen, San Diego, CA), 0.25 µM each primer, and ~ 0.14 µg of cDNA. The conditions for the allele-specific amplification were as follows: hot start, 94°C for 2 min; denaturation, 94°C for 90 s; annealing, 59°C for 60 s; extension, 72°C for 30 s for 35 cycles with an 8 min extension on the final cycle. The PCR products were analyzed by 2% (w/v) agarose gel electrophoresis.

The reaction mix for the first round of the nested PCR contained: 1x PCR buffer I (see above), 10 pmol of each primer, 200 µM each dNTP, 1.5U AmpliTaq® DNA polymerase and 10µl of the cDNA template. The cDNA samples were heated at 94°C for 5 min and then amplified for 35 cycles of: denaturation, 94ºC for 1 min; annealing, 58ºC for 1min; extension, 72ºC for 2 min, with a final extension at 72°C for 12 min. For the second round of nested PCR a variable number of PCR cycles were used with the following conditions: hot start, 94°C for 5 min; denaturation, 94°C for 1 min; annealing, 60ºC for 1 min; extension, 72ºC for 2 min, with a final extension at 72ºC for 30 min. RT-PCR products from the nested PCR reaction were qualitatively analyzed by fragment analysis in an automatic sequencer [58].

Western Blot

The 6CFSMEo- clone, grown as described above, was analyzed for CFTR protein expression by Western blot (WB) analysis [59]. Briefly, cells were lysed with sample buffer [1.5% (w/v) SDS (sodium dodecyl sulphate), 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue, 0.5mM dithiothreitol and 31.25mM Tris, pH 6.8], and the total protein was quantified by a modified micro Lowry method. Aliquots (250 µg) of total protein were loaded onto a SDS-polyacrylamide mini-gel (7% (w/v) polyacrylamide) (BioRad, Hercules, CA, USA) for electrophoretic separation. The protein in the gel was transferred to Protran® nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany). Membranes were probed

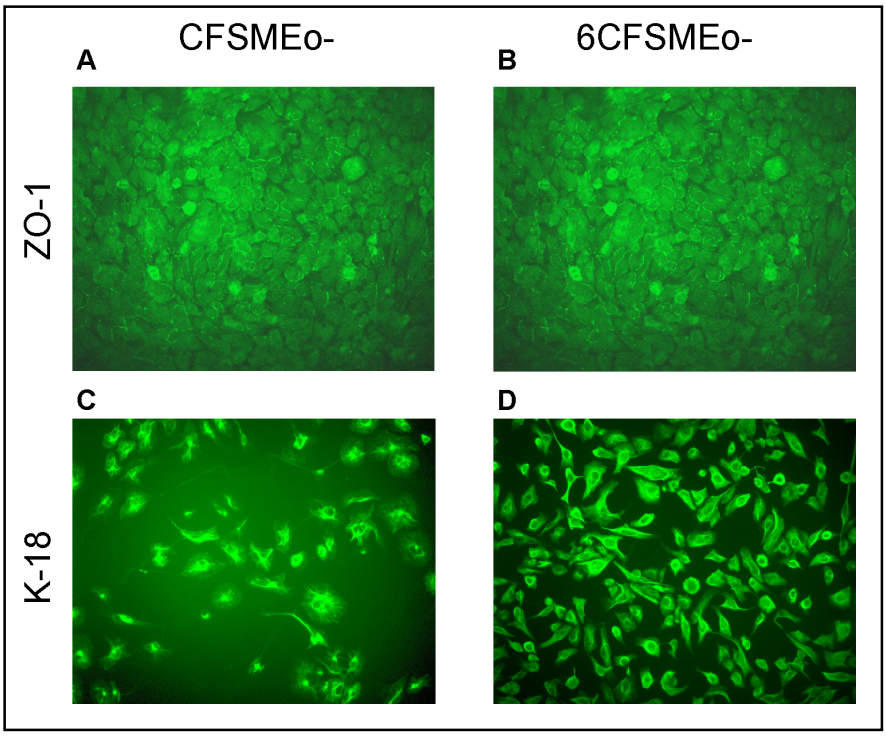

Fig. 1. Immunocytochemistry: Cells were stained with FITC-tagged primary antibodies K18 and ZO-1. Panels A and C show staining of CFSMEo- cells with the K18 and ZO-1 Abs, respectively. Panels B and D show the staining of the 6CFSMEo- with the same Ab as indicated. Staining for K18 indicates a well-organized keratin filament structure observed in primary airway epithelial cells. The localization of ZO-1 to the plasma membrane at points of cell-cell contact is expected and consistent with the formation of tight junctions and the maintenance of cell polarity. As expected, both cell lines were also positive for staining with Ab for the SV40 large T antigen (data not shown).

with the M3A7 antibody (Ab) (Chemicon, Temecula, CA, USA), previously shown to specifically detect CFTR [60], and a secondary anti-mouse, peroxidase-labeled monoclonal Ab (Amersham Bioscience, Piscataway, CA, USA). Blots were developed using the ECL™ detection system (Amersham Bioscience).

CFTR Immunoprecipitation

Expression of CFTR protein was also characterized in the 6CFSMEo- cells by immunoprecipitation (IP) [61]. Briefly, the cells were starved for 30 min in methionine-free MEM and then pulsed for 3 h in the same medium supplemented with 140 µCi/ml [^{35}S] methionine. Cells were then lysed in 1ml of RIPA buffer (1% (w/v) deoxycholic acid, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl). IP was carried out using the M3A7 Ab. Immunoprecipitated proteins were mixed protein G agarose beads and eluted with sample buffer at room temperature for 1h**.**

Patch Clamp Experiments

Cell culture dishes were mounted on the stage of an inverted microscope (IM35, Zeiss, Oberkochen, Germany) and kept at 37°C. The bath was continuously perfused with Ringer solution at a rate of about 10 ml/min. Patch clamp experiments were performed in the fast whole cell configuration as described [62]. The patch pipettes had an input resistance of 2-4 MΩ when filled with a solution containing (mM): KCl 30; K-gluconate 95; NaH_2PO_4 1.2; Na_2HPO_4 4.8; EGTA 1, $CaCl_2$ 0.726; $MgCl_2$ 1.034; D-glucose 5; ATP 3 (32 Cl). The pH was adjusted to 7.2; the Ca^{2+} activity was 0.1 µM. The access conductance was measured continuously and was between 30 and 120 nS. Currents (voltage clamp) and voltages (current clamp) were recorded using a patch clamp amplifier (EPC 7, List Medical Electronic, Darmstadt, Germany) and data were stored continuously on a computer hard disc. Cells were voltage clamped between –50 and +50 mV. At regular intervals, membrane voltages (V_m) were clamped in steps of 10 mV from -50 mV to +50 mV, and G_m was calculated from the measured I and V_m values according to Ohm's law [62].

Results

Immunocytochemical Analysis

Both the CFSMEo- and 6CFSMEo- cells retain the characteristic "cobblestone" appearance of epithelial cells. This is further confirmed by immunocytochemical staining of well-organized cytokeratin filaments with the airway epithelial-specific K18 antigen (Figure 1). Staining with monoclonal antibodies to the zonula occludens molecule, ZO-1 showed localization of this molecule, a component of the zonula adherens, to the cell periphery. The presence and localization of the ZO-1 is indicative of an intact junctional complex and is characteristic of the cell-cell contacts associated with tight junctions.

Mutation Analysis

Analysis of genomic DNA from 6CFSMEo- cells by sequencing of the CFTR coding regions and 3' trailer,

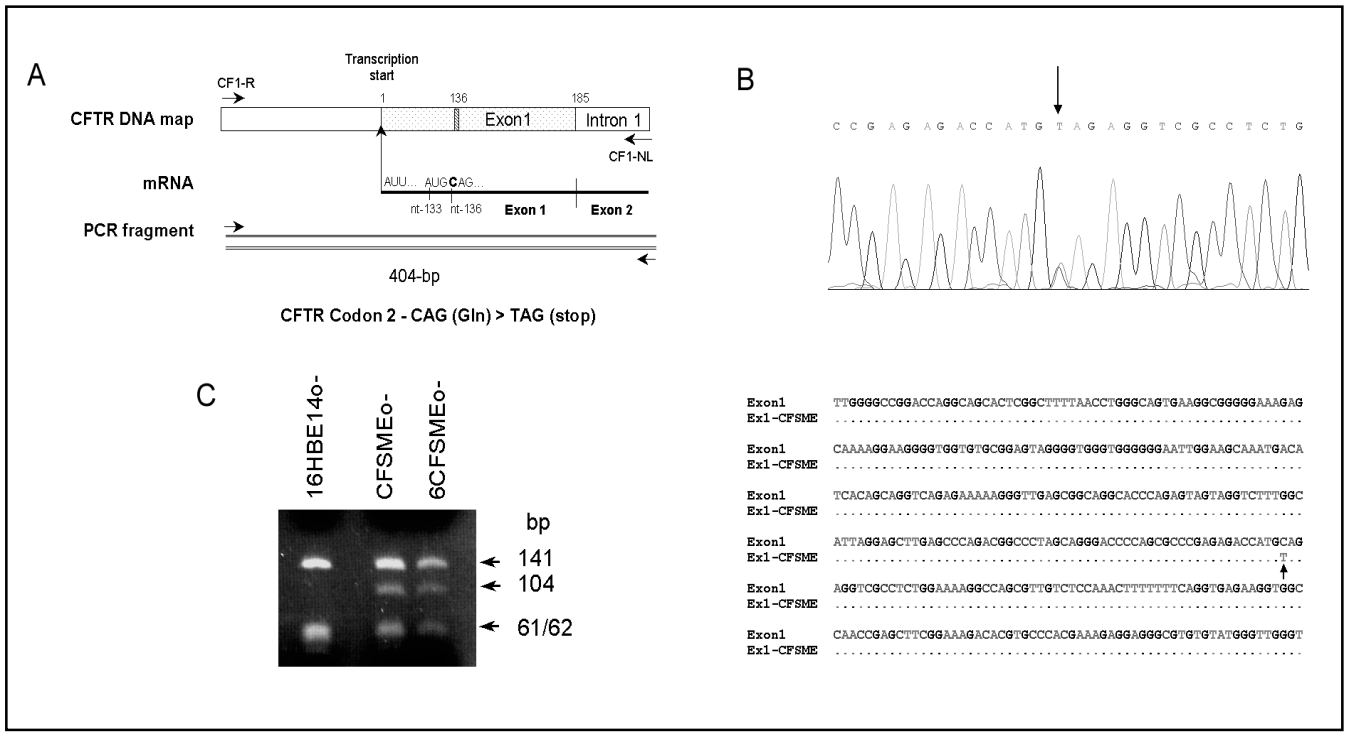

Fig. 2. Q2X Mutation (Exon 1) Analysis. (A) Schematic representation of the strategy used for mutation analysis in the region of exon 1 of the CFTR gene. (B) Results from automatic sequencing of CFTR exon 1 PCR products (see Methods). The presence of a C>T mutation at nt 136 of exon 1 is pointed with an arrow. This transition mutates the second codon (CAG, glutamine) into the stop codon UAG, being thus termed Q2X (C) Mwo I RFLP analysis: PCR amplification of CFSMEo-, 6CFSMEo-, and 16HBE14o- (control) DNA with primers CF1-R and CF1-NL. Amplicons were cut by the Mwo I restriction enzyme. For the normal sequence, Mwo I generates bands at: 15-24-42-53-61-62-141-bp. For the mutant sequence, bands are generated at: 15-24-53-61-104-141-bp. The smaller bands are not readily visualized on the gel. The 16HBE14o- cells will not generate a 104-bp band as indicated above.

confirmed presence of the F508del mutation in exon 10 (data not shown) and revealed the presence of a T>C mutation at nt 136 of exon 1 (Fig.2). This transition mutation converts the second codon (CAG, glutamine) into the stop codon UAG, Q2X, previously described CFTR gene variant [5].

CFTR Transcript Analysis

As a first approach to detect the presence of CFTR transcripts in both the CFSMEo- and the 6CFSMEo- cell lines, allele-specific RT-PCR analysis was performed in the region of exon 10 (Fig 3A). Unlike what was observed for the 6CFSMEo- cell line (and now the CFSMEo- cell line) no CFTR mRNA expression was detected from either allele. A more sensitive RT-PCR analysis of the region encompassing exons 8 through 10 with a fluorescent primer (Fam-labeled) [58], also failed to detect any products (data not shown). To further enhance the sensitivity, nested RT-PCR was performed in the region comprising CFTR exon 7 through 13 (first round), and 8 through 10 (second round). The products were analyzed in an automated sequencer (see Methods) that differentiates the F508del products from those of the other (non-F508del) allele as a result of the 3-bp size difference [58]. Two major peaks were observed (Fig.3B): one, at 388-bp resulting from the F508del transcripts and another, at 391-bp, corresponding to the Q2X mRNA. It has been generally observed that the F508del-transcripts are less abundant than those from the wt-CFTR allele [58], here we find that the Q2X-transcripts are present at levels lower than the F508del-transcripts. This finding is particularly instructive in light of both the stop mutation in

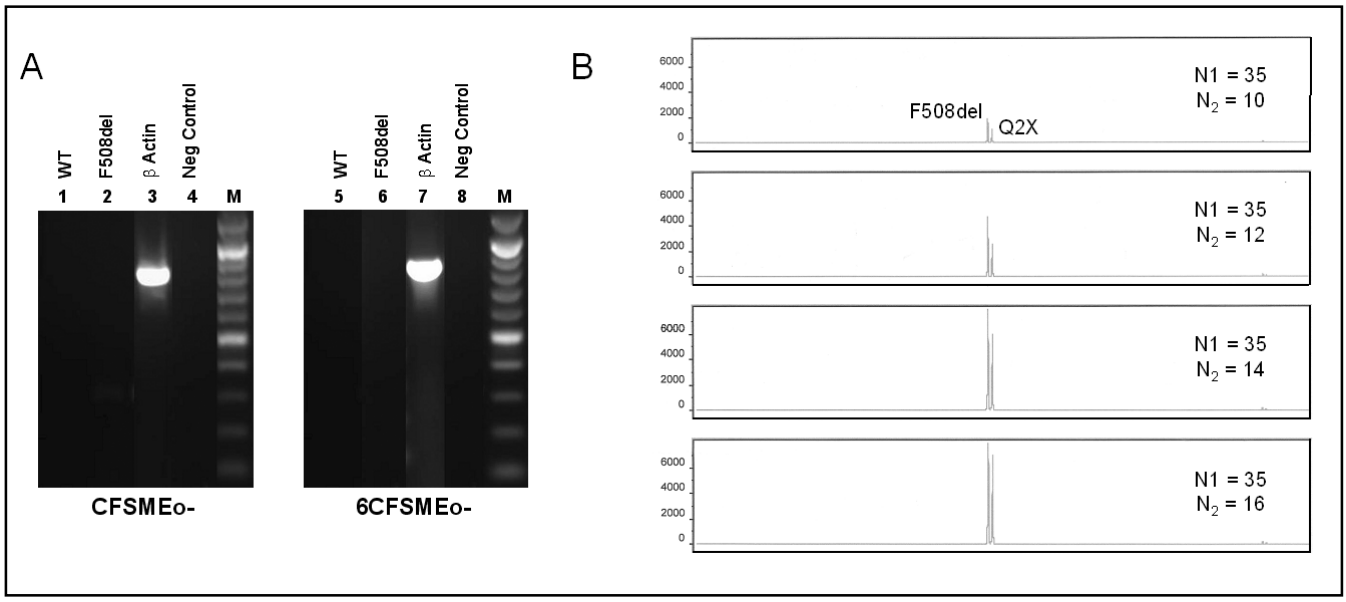

Fig. 3. RT-PCR Analysis. (A) Standard agarose gel analysis of products from allele-specific RT-PCR amplification (exons 9-10) showed no product for either allele after 35 cycles for the CFSMEo- (left panel) or the 6CFSMEo- (right panel) cells. (B) RT-PCR products obtained after nested PCR (i.e., 2 rounds) using one Fam-labeled primer were analyzed by a capillary electrophoresis automatic sequencer. The first round amplification (N1=35 cycles) was carried out of the region encompassing exons 7 through 13 (no products were detected, data not shown). A second round (nested) of amplification of the region encompassing exons 8 through 10 was carried out for a variable number (N2) of cycles (as indicated in each elecropherogram). RT-PCR products from the F508del (388-bp) and the Q2X (391-bp) alleles can be well resolved using this method [58]. Although this method is not quantitative when applied to nested PCR, an integration of peak areas corresponds to relative amounts of the F508del and Q2X products. The Q2X product is estimated to be roughly 50% of the F508del when the PCR is kept within the exponential phase of the amplification (i.e., $N_2 < 14$ cycles). The elecropherogram scale at the left is in arbitrary density units.

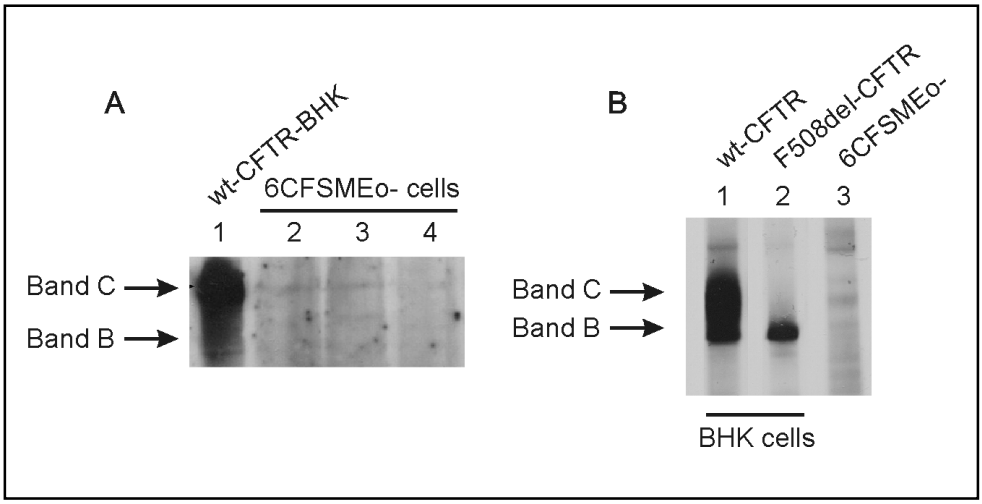

Fig. 4. CFTR Protein Analysis. Detection of CFTR in 6CFSMEo- cells by Western Blot (WB) analysis (A) with the anti-CFTR M3A7 Ab in (from left to right): lane 1, control BHK cells over-expressing wt-CFTR; lanes 2-4, three distinct protein samples from 6CFSMEo- cells. (B) Detection of CFTR by in vivo radio-labeling followed by anti-CFTR immunoprecipitation (IP) in: control BHK cells overexpressing, wt-CFTR (lane 1) or F508del-CFTR (lane 2); and lane 3, 6CFSMEo- cells. Arrows indicating bands B, and C refer to the core- (150 KDa) and fully-glycosylated (170-180 KDa) forms of CFTR, respectively.

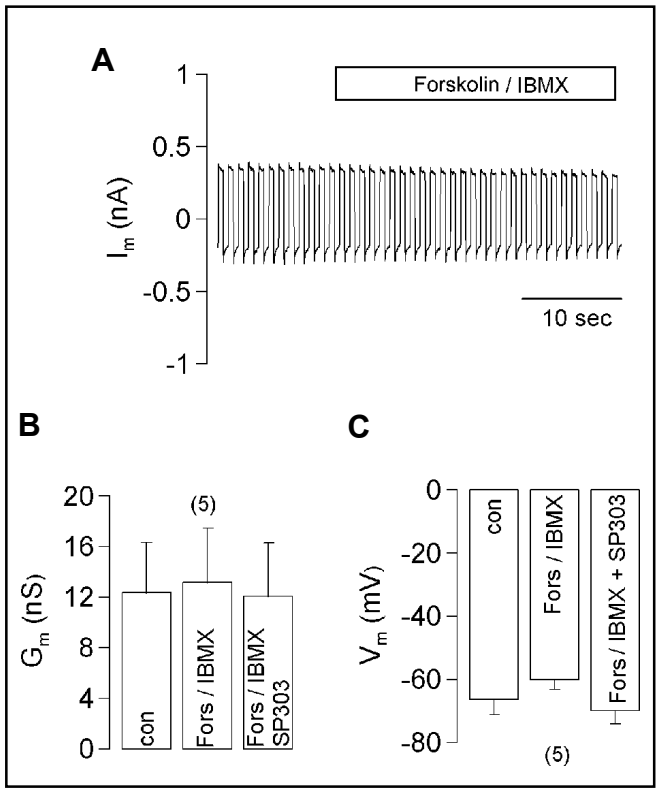

Fig. 5. Lack of cAMP activated Cl^- currents. (A) Fast whole cell patch clamp analysis. Original recording of the whole cell current from a 6CFSMEo- cell. Stimulation with forskolin (2 µmol/l) and IBMX (100 µmol/l) does not change the whole cell current. (B,C) Summaries of the calculated whole cell conductances show no effect on increased cAMP levels or of SP-303 on whole cell conductance (G_m) and membrane voltage (V_m). (number of experiments).

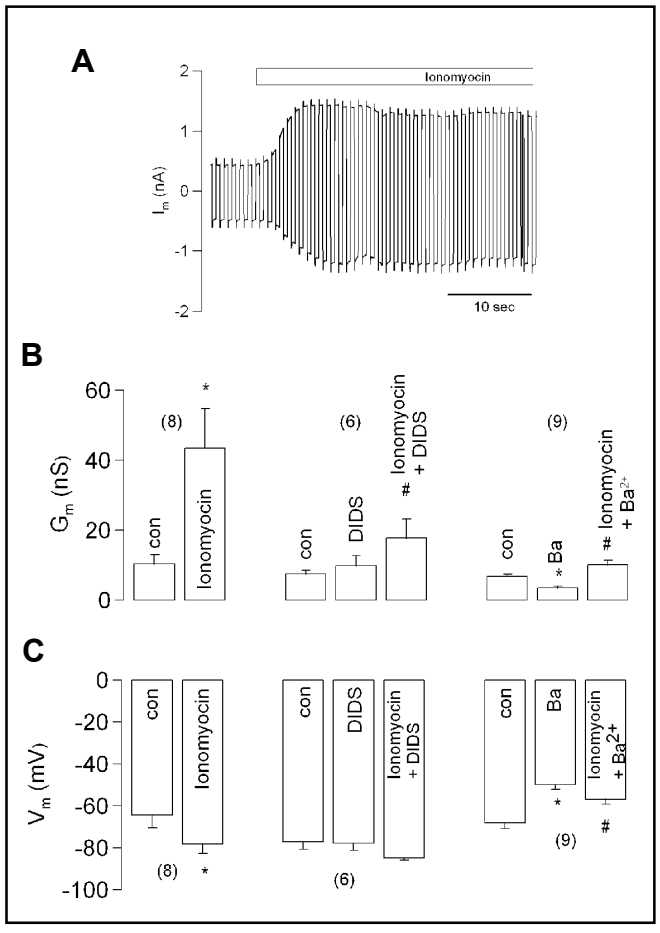

Fig. 6. Ca^{2+} activated Cl^- and K^+ currents. (A) Fast whole cell patch clamp analysis. Original recording of the whole cell current from 6CFSMEo- cells. Stimulation with ionomycin (1 µmol/l) activates a whole cell current. (B,C). Summaries of the calculated whole cell conductances (G_m) and membrane voltages (V_m) indicate that ionomycin activates a whole cell conductance that is partially inhibited by both DIDS (100 µmol/l) and Ba^{2+} (5 mM). Ionomycin hyperpolarizes V_m, while Ba^{2+} significantly depolarizes V_m. *, # indicate significant difference for the control and for preincubation with DIDS and Ba^{2+}, respectively. (number of experiments).

exon 1 and the reduction of the F508del mRNA expression compared to a previous analysis [30]. Although the method used here to analyze CFTR transcripts was originally described as quantitative [58], quantification of the Q2X mRNA levels cannot be accurately performed, due to the limitations inherent to the nested PCR amplification. Nevertheless, Q2X transcripts are roughly estimated to be about 50% of F508del mRNA levels in the 6CFSMEo- cells (Fig.3B, second panel from the top). Furthermore, the reduction in the F508del CFTR mRNA relative to the previous study is not unexpected, since changes in gene expression are often a function of the length of time in culture.

CFTR Protein Analysis

CFTR expression at the level of the protein was also characterized in the 6CFSMEo- cells by Western blotting using a CFTR-specific Ab (Fig.4). A protein sample from a BHK cell line expressing wt-CFTR in high amounts [45] was used as a positive control (Fig.4A). In several distinct samples from the 6CFSMEo- cells, no CFTR protein could be detected, even though it was clearly detectable in the control (Fig.4A). To increase the sensitivity of this analysis, ^{35}S-methionine labeled proteins were immunoprecipitated with the CFTR antibody, using samples from BHK cells expressing either wt- or F508del-CFTR as controls (Fig. 4B). CFTR was clearly detected in the overexpressing BHK cells (wt-,

TREATMENT	G_m Basal (nS) ± SEM	V_m Basal (mV) ± SEM	G_m Activated (nS) ± SEM	V_m Activated (mV) ± SEM
Forskolin/IBMX	8.10±1.41	-43.14±8.10	8.17±1.47	-41.29±7.67
Ionomycin	8.00±0.80	-55.00±2.00	21.40±1.80	-69.40±3.1
DIDS	8.68±0.65	-49.00±4.95	7.80±0.65	-54.00±3.96
IONO/DIDS	8.68±0.65	-49.00±4.95	15.38±1.03	-73.60±3.80
Barium	7.28±0.42	-55.17±2.36	4.23±0.30	-26.33±2.20
IONO/Ba	7.28±0.42	-55.17±2.36	6.05±0.40	-31.50±2.45

Table 2. Electrophysiological Analysis of CFSMEo- Cells. Legend: Electrophysiological responses of CFSMEo- cells to treatments with various agonists. Changes in membrane conductance (G_m) and in membrane potential difference (V_m) as a function of treatment with forskolin/IBMX (n=7), ionomycin (n=7), DIDS (n=5), Barium (n=6), and Ionomycin plus DIDS (n=5) or ionomycin plus barium (n=6). The experiments with barium with or without ionomycin and DIDS with or without ionomycin were conducted by sequentially adding ionomycin to either the barium or the DIDS accounting for the same basal G_m) and V_m). SEM=standard error of the mean.

band C and band B or F508del-CFTR, band B). However, there were no clearly discernable CFTR-specific bands in the region of 150 KDa (band B) or 170-180 KDa (band C) in the samples from 6CFSMEo- cells.

Electrophysiological Properties of Cells

The electrophysiological properties of the 6CFSMEo- cell line were determined using the patch clamp fast whole cell technique. Stimulation of cells with forskolin (2 μM) and IBMX (100 μM) did not activate a whole cell current, indicating a lack of functional CFTR (Fig. 5A). Moreover, application of the CFTR Cl^- channel blocker, SP-303 (1 μM) [63] in the presence of forskolin and IBMX had no effect on the whole cell conductance (Fig. 5B,C). In contrast, when intracellular Ca^{2+} was increased by ionomycin, whole cell current and conductance were increased significantly (Fig. 6A,B). Ionomycin-activated whole cell conductance was inhibited by both the inhibitor of Ca^{2+} activated Cl^- channels, DIDS (100 μM) as well as the K^+ channel blocker, Ba^{2+} (5 mM). This indicates simultaneous activation of both Ca^{2+} activated Cl^- and K^+ channels in 6CFSMEo- cells by ionomycin. Stimulation with ATP (100 μM), a ligand of purinergic receptors, and application of amiloride (10 μM) had no affect on whole cell currents or membrane voltage (data not shown).

As expected, electrophysiological characterization of the CFSMEo- cells showed a similar response to that observed for the 6CFSMEo- cells (Table 2). The CFSMEo- cells showed no appreciable change in membrane conductance (G_m) or membrane voltage (V_m) following treatment with forskolin (10 μM) and IBMX 100 μM) or DIDS (100 μM). Treatment of the cells with ionomycin (1 μM) alone showed a significant increase in the G_m and decrease in V_m. DIDS had no affect on the ionomycin response when the cells were treated with both agents concomitantly. However, when the cells were treated with barium (Ba, 5 mM) alone they showed a decrease in G_m and an increase in V_m. The addition of ionomycin had no affect on the response of the cells to Ba^{2+}.

Discussion

The need for a additional cellular models that can be used in parallel studies with the airway submucosal gland cell line Calu-3, inspired this further characterization of the CFSMEo- and the 6CFSMEo- cell lines [30]. While a previous study has also reported a CF submucosal gland cell line, CF-KM4 [64], the cell lines examined here provide a significant additional resource for the characterization of the molecular pathways underlying CF pathology both in terms of the uniqueness of the genotype and its phenotypic properties described here and previously [30].

The F508del mutation, previously described for these cell lines, was confirmed in these studies. In addition, a

second, rare CF-causing mutation, the Q2X nonsense mutation in codon 2, makes these cell lines unique in their genotype. While this mutation has been previously identified in another patient [5], there are no established cell lines of an identical genotype.

Assessment of CFTR expression at the level of mRNA was quantitatively approached using a highly sensitive method described previously [58]. CFTR mRNA in the CFSMEo- and 6CFSMEo- cells was initially measured by allele-specific RT-PCR [56] or by an RT-PCR analysis used to detect CFTR transcripts in native tissues from patients [58, 65], and no products were detected. However, after two rounds of PCR with at least 45 cycles of PCR amplification, CFTR transcripts could be detected from both the F508del and the Q2X alleles. The Q2X-transcripts were present at levels approximately 50% lower than F508del-transcripts. Enhanced degradation of transcripts containing premature termination (stop) codons (PTCs) is known to occur by a mechanism, termed nonsense-mediated decay (NMD) [66], and has been described for some CFTR alleles [67]. It is therefore highly likely that the Q2X transcripts are degraded due to NMD.

Despite the significant reduction in the Q2X-transcript levels, the use of an alternative initiation codon could be employed to generate a functional CFTR protein, as suggested by others [68]. To further investigate this point and to establish if CFTR protein from the F508del allele is produced by the 6CFSMEo- cells in appreciable, detectable levels, the cells were analyzed by both WB and IP. When compared to control samples, i.e., cells overexpressing wt- or F508del-CFTR, the results obtained for 6CFSMEo- cells were consistent with a lack of CFTR protein expression from either allele.

Examination of the functional characteristics of the CFSMEo- and 6CFSMEo- cells measured the response of the cells to cAMP or Ca^{2+} agonists. As expected, neither the CFSMEo- nor the 6CFSMEo- cells showed cAMP-dependent Cl^- transport upon stimulation with IBMX and forskolin. The whole cell current increased significantly when intracellular Ca^{2+} was released with ionomycin. Stimulation of secretion in non-CF submucosal cell lines by activation of Ca^{2+} dependent K^+ channels has been observed previously [69, 70]. This K^+ conductance is likely to be due to the Ca^{2+} activated K^+ channel SK4 [71, 72]. Interestingly, DIDS inhibitable Ca^{2+} activated Cl^- channels were not detected in the wt-CFTR expressing Calu3 cells, but may be present in freshly isolated submucosal glands from CF patients [69, 73]. While the identity of apical Cl^- secretory pathways is controversial, in normal submucosal gland cells, CFTR appears to be essential for Cl^- and HCO_3^- secretion. However, this doesn't exclude the possibility of other alternative Cl^- channels [3, 20]. The data present in this study indicate the presence of Ca^{2+} activated Cl^- channels that are present in submucosal gland cells from CF patients. Thus, the CFSMEo- and 6CFSMEo- cell lines provide an important tool for studying the pathways underlying Cl^- transport in submucosal gland cells.

The major objective in the establishment of cell culture systems for CF research is to provide *in vitro* models that resemble, as closely as possible, the properties of the native tissue from which they were derived. The CFSMEo- and 6CFSMEo- cell lines were immortalized from cultures enriched for airway gland epithelial cells [49], found to reproduce the phenotypic properties of their progenitors [30] and to maintain their tight junctions and cell polarity so that they can form tight monolayers on permeable supports (M. Amaral and DC Gruenert, unpublished observations). The characterization of CFTR expression and the ion transport properties of these cell lines demonstrate the lack of protein and the absence of cAMP-stimulated Cl^- currents. In addition to its unique genotype, these cell lines provide an ideal model for CF submucosal gland epithelial cells that have a similar histological origin as the wt-CFTR expressing, Calu-3 cells. Given our present understanding of CFTR expression patterns and CF pathology, these culture systems should thus be very useful to study the mechanisms underlying CF biology and pathology and may be an effective, CFTR-protein negative, control for a number of studies, including gene targeting.

Abbreviations

Ab (antibody); ASL (airway surface liquid); CF (cystic fibrosis); CFTR (CF transmembrane conductance regulator); IP (immunoprecipitation); NMD (nonsense-mediated decay); PTC (premature stop codon); SDS (sodium dodecyl sulphate); WB (Western blot); PCR (polymerase chain reaction); RT-PCR (reverse transcriptase-PCR).

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