Characterization of CFTR expression in a human pulmonary mucoepidermoid carcinoma cell line, NCI-H292 cells

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Abstract The NCI-H292 cell, a human pulmonary mucoepidermoid carcinoma cell line, is commonly used for studying bacterial and viral infections of airway epithelial cells. Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) is the main cause of fetal lung infection in cystic fibrosis patients. In this study, we examined CFTR expression in NCI-H292 cells to determine whether NCI-H292 cells possess sufficient, normally functioning CFTR. The results of RT-PCR and Northern blotting analysis indicated that the CFTR gene expression level was much lower in NCI-H292 cells than in T84 cells. However, Western blotting analysis showed that protein expression in NCI-H292 cells was comparable to that in T84 cells. Furthermore, whole-cell and cell-attached patch clamp electrophysiological techniques indicated that the Cl^- current induced by intracellular cAMP elevation in NCI-H292 cells was comparable to that in T84 cells. These findings suggest that NCI-H292 cells with a low level of CFTR gene expression possess enough functional CFTR to show a physiological response.

Key words: NCI-H292 cell line; Airway epithelial cell; T-84 cell line; Cystic fibrosis transmembrane conductance regulator; Cl^- channel; Lung infection

1. Introduction

Cystic fibrosis (CF) is a recessive genetic disorder, which affects about 1 in 2500 Caucasian live births [1]. The morbidity and death from CF result mainly from complicating pulmonary infections and inflammation.

The NCI-H292 cell, a human pulmonary mucoepidermoid carcinoma cell line, is commonly used for studying bacterial and viral infections and inflammation in airway epithelial cells [2–7]. For example, *Pseudomonas aeruginosa* lipopolysaccharide profoundly upregulates transcription of the mucin gene in NCI-H292 cells [4], and influenza A virus infection upregulates interleukin (IL) 6, IL-8, and RANTES expression in NCI-H292 cells [5]. The NCI-H292 cell is sensitive to the cytotoxic activity of *Bordetella bronchiseptica* [6]. *Haemophilus influenza* passes through viable layers of NCI-H292 cells by paracytosis, which requires bacterial protein synthesis [7]. As far as we know, this cell line is a good model for studying the pathological changes in airway epithelial cells. It seems to us that NCI-H292 cells readily permit the infections and inflammation seen in CF patients. However, there is no information on CFTR expression in these cells, although CFTR function is closely tied to infectious inflammation in airways. Therefore, in this study, we examined CFTR gene expression, protein expression, and the functional response of the Cl^- channel.

2. Materials and methods

2.1. Cell culture

NCI-H292 (a human pulmonary mucoepidermoid carcinoma cell line) and T84 (a colon carcinoma cell line) were purchased from the American Type Culture Collection (Rockville, MD, USA). NCI-H292 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. CFTE29o− and CFBE41o− (an immortalized cystic fibrosis tracheal and bronchial epithelial cell line) was obtained from D.C. Gruenert. T84, CFTE29o− and CFBE41o− were grown as previously described [8,9].

2.2. Northern blot analysis

A single-step method of isolating RNA using acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi [10] was used to isolate total cellular RNA from cells grown in 75 cm^2 tissue culture flasks. Twenty micrograms of total RNA per lane were electrophoresed on formaldehyde-agarose gels, blotted onto nylon membranes, and baked in an oven for 2 h at 80°C. For hybridization with the cDNA probe, which was the nested PCR product described below, the blots were prehybridized in 5×SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.005 M EDTA), 10×Denhardt's solution, 50% formamide, 2% sodium dodecyl sulfate (SDS), and 100 μg/ml of denatured salmon sperm DNA at 42°C. The cDNA probes were labeled with [α-^{32}P]dCTP using Amersham's random primer labeling kit, and hybridization was performed under the same conditions, using probes with $>2\times10^6$ counts min^{-1} ml^{-1}. A series of 20 min washes was then performed, starting with 2×SSC (0.3 M NaCl and 0.03 M sodium citrate) at room temperature, followed by 0.5×SSC at 42°C, 0.2×SSC at 42°C, and if necessary, 0.1×SSC at 42°C. All the wash buffers contained 0.1% SDS.

2.3. RT-PCR

Total RNA was extracted from cell lysate, and cDNA was synthesized using virus reverse transcriptase and random hexanucleotide primers. CFTR transcripts were amplified by the polymerase chain reaction (PCR) using *Taq* DNA polymerase and the CFTR primers [11] (exon 9, HCF21: 5′-TTGCTGGATCCACTGGAGCAGG-3′; exons 12–13, HCF22: 5′-GCCATCAGTTTACAGACACAG-3′) under standard conditions, with an annealing temperature of 55°C. 'Nested' amplification was performed on the products of the first PCR reaction under similar conditions, using primers located in exon 10 (HCF30: 5′-CTTCACTTCTAATGATGATTATGG-3) and exon 11 (HCF31: 5′-TCTTTCTCTGCAAACTTGGAGATG-3′).

2.4. Western blot analysis

Cells grown to 100% confluence in 75 cm^2 tissue culture flasks were rinsed twice with ice-cold phosphate buffered saline (PBS). The cells were harvested with a cell scraper in ice-cold PBS and centrifuged at

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate; Mg-ATP, adenosine 5′-triphosphate magnesium salt

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1000 rpm for 10 min at 4°C. The pellets were lysed with 3× the pellet volume of lysis buffer (50 mM Tris containing 1% (w/v) *n*-octyl-β-D-thioglucopyranoside, 0.1% (w/v) sodium deoxycholate, 150 mM NaCl, 10 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, pH 7.5. After centrifugation (12 000 rpm for 10 min at 4°C), the supernatants were collected as lysate samples. The protein content of each fraction was measured by the BCA method [12] and the samples were stored at −80°C until required. All the samples were separated electrophoretically by 7.5% SDS-PAGE in non-reducing conditions (100 μg protein/lane) according to the method of Laemmli [13]. After electrophoresis, the proteins were transferred to PVDF membranes (Millipore Corp., Japan) by applying 250 mV for 90 min. The membranes were incubated with 5% non-fat dry milk in 0.1% Tween 20 in PBS at 4°C overnight. Then the membranes were washed in PBS containing 0.05% Tween 20 (PBS-T). Primary antibodies (0.2 mg/ml) were dissolved in PBS-T. The membranes were incubated for 1 h with monoclonal antibody against the CFTR protein C-terminal (Genzyme Inc., USA) (1:500 dilution in PBS-T), washed in PBS-T, and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG as the secondary antibody (Jackson ImmunoResearch Laboratories Inc., USA) (1:10 000 dilution in PBS-T). They were washed in PBS-T again and the blots were developed in enhanced chemiluminescence reagent (ECL kit, Amersham, UK) and visualized on X-ray film after a 1–15 min exposure.

Calnexin was used as a loading control. Calnexin was detected by polyclonal antibody against the calnexin protein C-terminal (Stress-Gen Biotechnologies Corp., Canada) (1:1000 dilution in PBS-T containing 5% skim milk powder). The secondary antibody was HRP-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc., USA).

2.5. Patch-clamp analysis

Whole-cell and single-channel currents were recorded as described previously [14,15], using an Axopatch-1C amplifier (Axon Instruments, USA). All the patch-clamp recordings were made at room temperature.

For whole-cell experiments, the pipette solution contained 140 mM CsCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM glucose, 2 mM Mg-ATP, and 5 mM HEPES (pH 7.35 adjusted with 298 mosm kg^{-1} CsOH). The bath solution contained 170 mM Tris-HCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM HEPES, and 10 mM glucose (pH 7.4, 325 mosm kg^{-1}). For the whole-cell experiments, the patch pipettes had resistances of 4–6 MΩ. The membrane was voltage-clamped to a holding potential of −70 mV and stepped to levels between −110 mV and +110 mV at 55 mV intervals. The whole-cell currents were filtered at 1 kHz, and digitized.

For single-channel experiments with cell-attached patches, the pipette solution contained 150 mM *N*-methyl-D-glucamine chloride, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 10 mM HEPES. The bath solution contained 150 mM NaCl, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 10 mM HEPES (pH 7.3 adjusted with NaOH, 300 mosm kg^{-1}). For single-channel experiments, the patch-pipettes had resistances of 10–12 MΩ. The single-channel currents were filtered at 1 kHz, digitized at 100 Hz, and analyzed using pClamp software (Axon Instruments, USA).

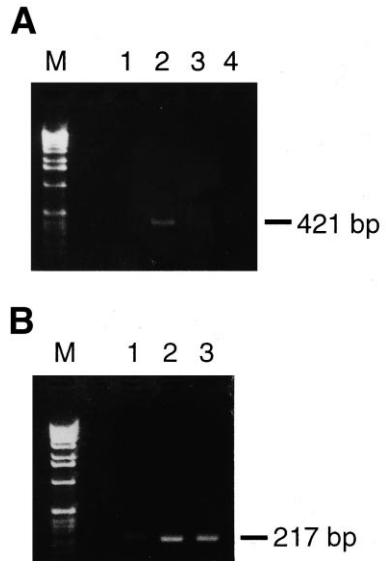

Fig. 2. Reverse transcriptase PCR analysis for the presence of CFTR mRNA in human epithelial cell lines. A: The cytoplasmic RNA was converted to first-strand cDNA and then amplified with primers at the 5′ and 3′ ends of exons 9 and 12–13. The predicted fragments of 421 bp for CFTR mRNA were present. Lane M: DNA size marker, lane 1: NCI-H292, lane 2: T84, lane 3: CFTE29o−, lane 4: CFBE41o−. B: The RT-PCR products were nested-amplified with primers at the 5′ and 3′ ends of exons 9 and 10–11. The predicted fragments 217 bp for CFTR mRNA were present. Lane M: DNA size marker, lane 1: NCI-H292, lane 2: CFTE29o−, lane 3: CFBE41o−.

3. Results

Initially, the NCI-H292, CFTE29o− and CFBE41o− (from CF patients, homozygous for the CFTR delta F508 mutation), and T84 cells (a representative cell line with high expression of the CFTR gene [16]) were characterized for the expression of CFTR mRNA by Northern blot hybridization (Fig. 1). T84 cells expressed CFTR mRNA that was approximately 6.5 kb long, but the other cells did not. Next, the expression of CFTR mRNA was investigated by RT-PCR and its expression in T84 cells was confirmed by the presence of the predicted 421 bp fragment spanning exons 9 to 12–13, but the other cells did not (Fig. 2A). In the other cell lines, a 217 bp fragment spanning exons 10–11 was detected by nested PCR (Fig. 2B). Western blotting analysis of the protein extracted with an antibody against CFTR C-terminal indicated

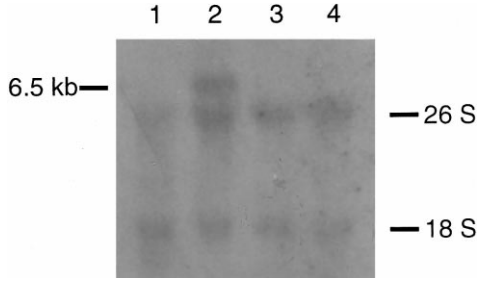

Fig. 1. Expression of CFTR mRNA in human epithelial cell lines. Autoradiographic analysis of a representative Northern blotting with ^{32}P-labeled CFTR probe. Total cytoplasmic RNA (20 μg/lane) was loaded in each lane. Lane 1: NCI-H292, lane 2: T84, lane 3: CFTE29o−, lane 4: CFBE41o−.

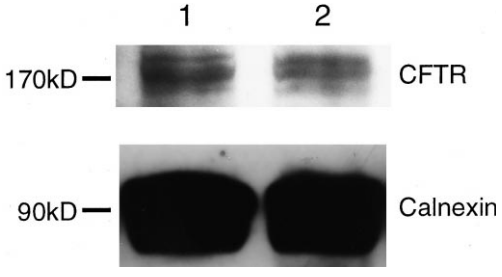

Fig. 3. Expression of CFTR protein in human epithelial cell lines. Total cellular protein (100 μg/lane) was loaded. Lane 1: NCI-H292, lane 2: T84. Immunoreactive proteins were detected with antibody against the CFTR protein C-terminal by enhanced chemiluminescence. Calnexin was used as a loading control.

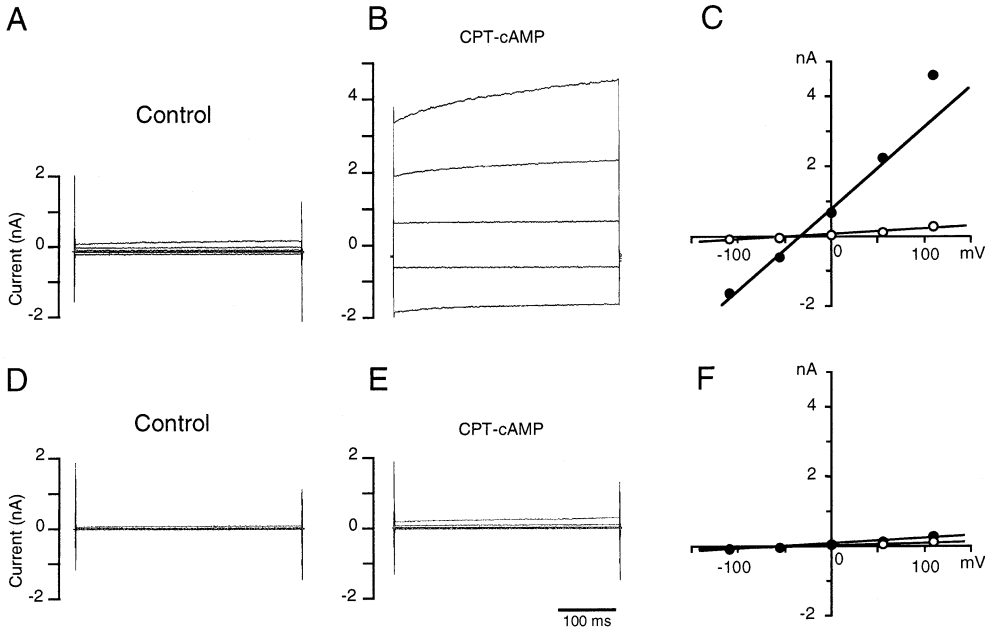

Fig. 4. Representative whole-cell current recordings from airway epithelial cells. A and B: Current recordings before and after the application of 1 mM CPT-cAMP in NCI-H292 cell. C: Corresponding current-voltage relationships of the current records shown in A and B. D and E: Current recordings before and after the application of 1 mM CPT-cAMP in CFTE29o− cell. F: Corresponding current-voltage relationships of the current records shown in D and E. Membrane voltage was held at −70 mV.

that cells produced a 170 kDa antigenic protein, comparable to that seen in protein extracted from T84 colon carcinoma cells (Fig. 3). This band was previously identified as CFTR protein [17–19].

To examine the expression of CFTR function, whole-cell Cl^- currents were tested in NCI-H292 cells. Large activated, cAMP-dependent Cl^- currents were observed in cells (Fig. 4A–C). The reversal potential of the CPT-cAMP-stimulated current was 30.8 ± 4.9 mV, which agreed with the theoretically predicted value of 27.9 mV. Generally, the current-voltage (*I-V*) relationship for the cAMP-stimulated whole-cell CFTR Cl^- current was linear (Fig. 4C). The *I-V* relationship in NCI-H292 cells was almost linear, although at 110 mV, an outwardly rectifying Cl^- current appeared, as reported previously in other airway epithelial cells [20].

Cell-attached patch recording was used to identify the channels that underlie the whole-cell Cl^- conductance described above. NCI-H292 cells possessed low-conductance channels stimulated by 50 μM forskolin (Fig. 5A). The stimulated channel activities were suppressed by 50 μM glibenclamide, a CFTR channel blocker [21] (data not shown). The *I-V* curve was linear and the conductance, measured as the slope conductance through 0 mV, was 9.89 pS (Fig. 5B). These properties of the cAMP-stimulated Cl^- channel in NCI-H292 cells were very similar to previously reported values for CFTR [15,22].

4. Discussion

Our results indicate that NCI-H292 cells with a low level of CFTR gene expression possess enough functional CFTR to produce a physiological response. NCI-H292 cells may belong to the second group described in a previous report [23]. This group includes epithelial cells, including carcinoma cell lines

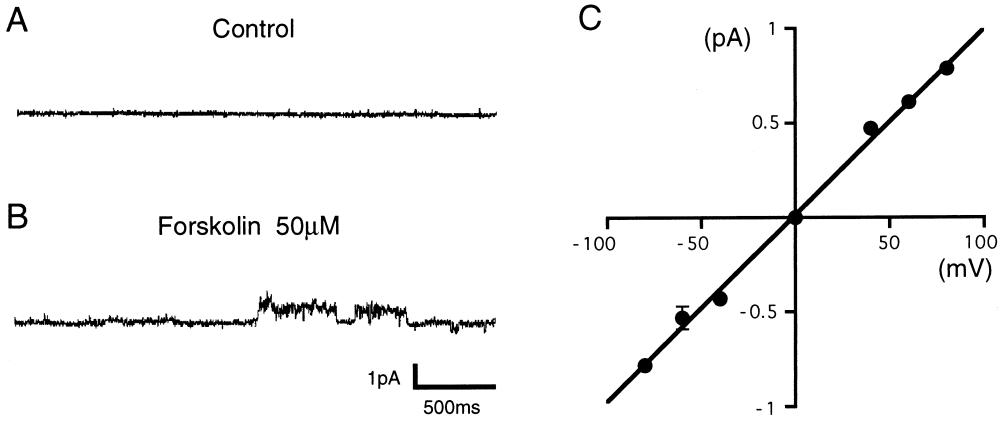

Fig. 5. Single channel currents in NCI-H292 cells. A and B: Single channel current recordings under cell-attached condition before and after the application of 50 μM forskolin in NCI-H292 cell. C: Corresponding current-voltage relationships of the forskolin-induced current records. $V_p = -40$ mV. Data are representative of seven experiments.

such as HeLa cervical carcinoma cells and HS-24 bronchial carcinoma cells [23], as well as transformed cell lines such as BET-1A bronchial cells and 293 kidney epithelial cells [23], which have lower levels of transcription and fewer mRNA transcripts. Interestingly, although the level of mRNA transcription in NCI-H292 cells was lower than that in T84 cells, the level of protein expression was almost the same. This level of protein expression was supported by electrophysiological data. At present, it is unclear whether the translation efficiency or protein stability is lower in T84 cells than in NCI-H292 cells, or if the CFTR translation machinery is already saturated in NCI-H292 cells.

The CFTR protein is a well-described regulator of epithelial ion transport and extracellular fluid composition, which acts at the outer cell surface. Recently, CFTR was identified as the bacterial ligand for ingestion in epithelial cell internalization in *Pseudomonas aeruginosa* [24]. Therefore, cystic fibrosis patients are susceptible to *P. aeruginosa*, because without CFTR on the epithelial cell surface this clearance mechanism cannot prevent bacterial colonization. NCI-H292 is commonly used to study bacterial and viral infection and inflammation in airway epithelial cells [2–7]. We initially assumed that CFTR function in NCI-H292 cells was low, facilitating bacterial infection. However, the present results indicate that NCI-H292 cells possess normal CFTR function. In CF, the loss of CFTR elevates the salt concentration in airway surface liquid and sweat by related mechanisms; the elevated NaCl concentration is due to a block in transcellular Cl^- movement. A high NaCl concentration may predispose CF airways to bacterial infection by inhibiting endogenous antibacterial defenses. Specific anti-bacterial proteins like β-defensins and lysozyme are inhibited at high NaCl concentrations [25]. Although we did not examine the expression of β-defensins and lysozyme in NCI-H292 cells, it is possible that NCI-H292 cells may express β-defensins and lysozyme at low levels.

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