30 min with 0.1 μM [^{3}H]IAA-94 (12 Ci/mmol). Bound ligand was separated from free by rapid gel filtration through a 0.5 cm by 3 cm G-50 Sephadex column, and the radioactivity present in the eluate was determined in a liquid scintillation counter to provide a measure of total binding. Nonspecific binding was defined as that occurring in the presence of 100 μM IAA-94, and the difference between total and nonspecific binding yielded specific [^{3}H]IAA-94 binding.

6. Chloride channels were reconstituted by a detergent dilution method [M. Kasahara and P. C. Hinkle, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 396 (1976); W. P. Dubinsky and L. B. Monti, *Am. J. Physiol.* **251**, C713 (1986)]. Solubilized proteins (see legend to Fig. 2) were diluted tenfold into excess asolectin (12 mg/ml) and frozen in an acetone-dry ice bath. The mixture was thawed at room temperature and sonicated in a bath-type sonicator for 25 s; external Cl^- was removed from the vesicles by passing them through an anion exchange column. Uptake of $^{36}Cl^-$ into the liposomes was measured at a final Cl^- concentration of 5 mM (2 μCi/ml), and uptake was stopped by passing the vesicles through a second anion exchange resin.
7. The concentration of eluted proteins was below the resolution of standard protein determination assays. On the basis of silver staining of known quantities of protein standards in the same gels, we estimated that for each preparation less than 1 μg of protein was present in the column eluate. Although silver staining is poorly quantitative, this estimate was supported by NH_2-terminal amino acid analysis of the 27-kD protein, which showed the presence of about 100 ng of protein.
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14. The purified channels were reconstituted into phospholipid vesicles by a detergent dialysis procedure. The vesicles were formed by concentrating 7 ml of the affinity column eluate to 1 ml with a PM-10 filter (Amicon, Danvers, MA) that had been coated with a 1% fibrinogen solution. The concentrated material was added to 10 mg of asolectin [Y. Kagawa and E. Racker, *J. Biol. Chem.* **246**, 5477 (1971)] and 9 mg of *n*-octyl glucoside, vortexed, placed in a dialysis tube (Spectra/por, Spectrum Medical Industries, Los Angeles, CA; 14-kD cutoff), and dialyzed for 15 hours against 1 liter of a solution containing 10 mM KCl, 700 mM sucrose, and 10 mM Hepes (*p*H 7.0). The dialyzate was changed and dialysis was continued for an additional 3 hours. Vesicles were stored on ice until use. Planar bilayers (4% asolectin in decane) were formed by the brush technique in a 100-μm hole [A. Finkelstein, *Methods Enzymol.* **32**, 489 (1974)] in a Teflon partition. Vesicles were squirted at the membrane through a micropipette positioned 20 to 50 μm from the planar bilayer [M. H. Akabas, F. S. Cohen, A. Finkelstein, *J. Cell Biol.* **98**, 1063 (1984); W. Niles and F. S. Cohen, *J. Gen. Physiol.* **90**, 703 (1987)]. The cis (vesicle-containing) chamber contained either 350 mM KCl, 10 mM $CaCl_2$, and 10 mM Hepes (*p*H 7.0) or 150 mM KCl, 400 mM urea, 20 mM hemicalcium gluconate, and 10 mM Hepes (*p*H 7.0). The trans side contained either 100 mM KCl, 20 mM hemicalcium gluconate, and 10 mM Hepes (*p*H 7.0) or 10 mM KCl, 20 mM hemicalcium gluconate, and 10 mM Hepes (*p*H 7.0) but without urea. The single-channel currents were amplified with a current-to-voltage converter and recorded on a PCM video tape recorder (Indec Systems, Sunnyvale, CA). The data were digitized and analyzed with the use of interactive programs on a laboratory computer system (Indec Systems). Records were filtered at 300 Hz before digitization through an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Potentials given are those in the cis chamber relative to virtual ground in the trans chamber. Selectivity ratios were calculated by means of the Goldman-Hodgkin-Katz equation with activity coefficient corrections.
15. Light-induced $^{36}Cl^-$ uptake was measured by adding 6 mM $^{36}Cl^-$ (2.3 μCi/ml) to thawed and sonicated liposomes in a glass fluorometric cuvette. The vesicles were exposed to light from a Kodak 650H slide projector. Samples (80 to 120 μl) were removed at the indicated times, applied to an 8-cm anion exchange column, and eluted with 1 ml of a solution containing 250 mM sucrose and 10 mM imidazole (*p*H 7.0) to remove extravesicular $^{36}Cl^-$. Uptake in the dark was measured from identical samples of each preparation and was found to be linear with time, and this uptake was subtracted from the light-dependent flux. The individual values were within 10% of the reported averages.
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Persistence of Abnormal Chloride Conductance Regulation in Transformed Cystic Fibrosis Epithelia

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An airway epithelial cell line (CF/T43) was developed by infecting cultured airway epithelial cells from patients with cystic fibrosis (CF) with the pZIPneoSV(X)1/SV40T retrovirus and selecting for G418 resistance and ion transport properties. The distinctive chloride secretory phenotypes of the CF cell line CF/T43 and a normal cell line (NL/T4) were not perturbed by SV40T-induced cell transformation. Epithelial cell lines generated from CF cells with the SV40T gene can be used to test candidate CF genes and to evaluate the molecular mechanisms responsible for the CF phenotype.

CYSTIC FIBROSIS (CF) IS AN AUTOSOmal recessive disease that affects epithelia of the airways, sweat glands, pancreas, and other organs. Abnormal regulation of transport proteins in the apical cell membrane, including the conductive Cl^- channel, is the direct effect of the abnormal gene (*1*). These abnormalities are exhibited in primary cultures of CF airway epithelial cells, but the limited availability of these cells currently restricts CF research. The gene encoding for the SV40 large T (SV40T)

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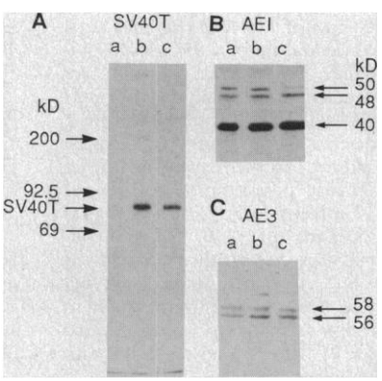

Fig. 1. (**A**) SV40T expression in CF/T43 and NL/T4 cell lines. Lane a, primary culture of normal airway epithelial cells; lane b, CF/T43; lane c, NL/T4. Cells were labeled with [^{35}S]-methionine (100 μCi/ml; >1000 mCi/mmol) (Amersham) in methionine-free Ham's F12 medium for 1 hour. The labeled cells were washed in phosphate buffered saline (PBS) and solubilized in lysis buffer (154 mM NaCl, 10 mM Na_2HPO_4, *p*H 7.2, 1% Triton X-100, 0.5% deoxycholate, and 1 mM NaF). The homogenate was centrifuged at 50,000*g* for 1 hour, and the supernatant (>10^7 tricarboxylic acid–precipitable counts per minute) immunoprecipitated with mouse monoclonal antibody 416 (Oncogene Science) to SV40T antigen (Ab-2a) according to the manufacturer's protocol (*16*). Immunoprecipitated antigen was analyzed by 8% SDS–polyacrylamide gel electrophoresis (*17*) followed by autoradiography. (**B**) Expression of AE1 cytokeratin and (**C**) expression of AE3 cytokeratin; the lanes are as in (A). Cells were collected by trypsinization and solubilized in sample buffer (62.5 mM tris-HCl, *p*H 6.8, 1% SDS, 2 mM phenylmethylsulfonylfluoride, 1 mM EDTA, 2.5 μg of leupeptin per milliliter, 2.5 μg of aprotinin per millilter, 5% glycerol, 50 mM dithiothreitol, and 0.001% bromophenol blue, *p*H 6.8). Proteins were separated by 8% SDS–polyacrylamide gel electrophoresis (*17*), and immunoblot analysis was performed (*18*). The reactivity of keratins with monoclonal antibodies AE1 and AE3 (*19*) was visualized by immunogold silver staining with Auro Probe BL plus streptovidin (Janssen Biotech, Olen, Belgium) according to the manufacturer's protocol.

antigen enhances the growth potential of cultured airway epithelial cells (*2*). To produce CF epithelial cell lines, we introduced the SV40T gene into primary cultures of CF and normal airway epithelial cells, selected clones that form resistive epithelial barriers, and characterized the regulation of their ion transport functions with intracellular microelectrodes and patch-clamp techniques. In this study we describe a CF epithelial cell line that expresses the abnormal regulatory phenotype.

Primary cultures of airway epithelial cells were established from nasal polyps excised from a donor with CF (diagnosed by elevated sweat Cl^-, typical airway disease, and pancreatic insufficiency) and from a proximal bronchus of an accident victim without CF (*3*). While in exponential growth phase, the cells were infected with a pZIP-neoSV(X)1/SV40T retrovirus (*4*). After 10 to 14 days, colonies resistant to G418 (a neomycin analog that is toxic to mammalian cells) (50 μg/ml) were isolated and propagated for further study, and samples were cryopreserved. Several cell lines were established, but most ceased proliferation after several passages. A CF (CF/T43) and a normal (NL/T4) cell line continued growing through 22 and 26 passages, respectively, and were used for further studies. Control cultures not exposed to the retrovirus senesced and died within four passages. The SV40T antigen was detected in the CF/T43 and NL/T4 lines, but not in primary nasal epithelial cell cultures (Fig. 1A), confirming expression of the exogenous gene. These cell lines expressed the same epithelial cell–specific cytokeratins as primary cell cultures (Fig. 1, B and C), confirming the epithelial nature of the cell lines.

The epithelial barrier and vectorial processes (such as transepithelial ion transport and secretion of glycoproteins) depend on development of intercellular tight junctions and cell polarization into distinct apical and basolateral regions (*5*). Because ion transport functions of the native tissue are best preserved in cell cultures that form resistive barriers (*6*), we refined culture conditions to induce formation of intercellular tight junctions and a transepithelial electrical resistance (R_t) in these cell lines. Cell lines were initially propagated in plastic tissue culture flasks in serum-free keratinocyte growth medium (KGM) (Clonetics). The cells were then passaged to permeable collagen matrix supports (CMS), the medium was supplemented with factors that induce differentiation in other cultured cells [1.0 m*M* Ca^{2+}, 10^{-9}*M* retinoic acid, and 50% Dulbecco's modified Eagle medium (with 2% fetal bovine serum) conditioned by NIH 3T3 fibroblasts (CM) (*6, 7*)], and R_t was measured serially in a sterile modified Ussing chamber. Cell lines with significant R_t's were evaluated by electrophysiological methods that distinguish normal and CF airway epithelial cells in primary culture (*8–10*).

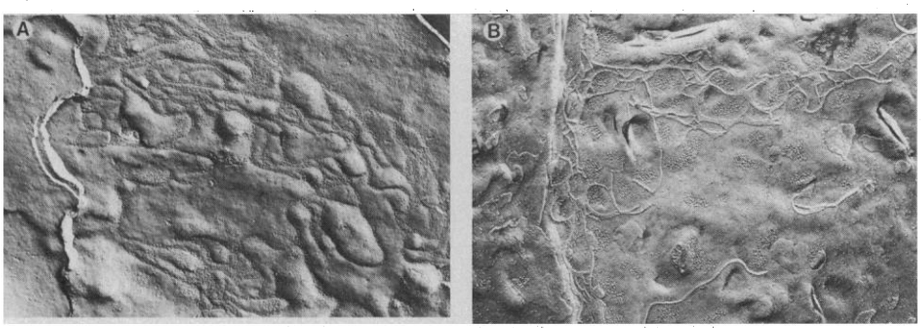

Fig. 2. (**A**) Freeze-fracture micrograph of CF/T43 cells showing E face grooves characteristic of tight junctions (×5500). (**B**) Normal airway epithelial cells in primary culture showing P face tight junction stands (×7100). CMS cultures were fixed in a mixture of phosphate-buffered 2% glutaraldehyde and 2% paraformaldehyde (*p*H 7.2). Before freezing, the samples were cryoprotected for 1 hour in a solution of 25% glycerol in 0.1*M* phosphate buffer. Samples were positioned on gold double-replica specimen mounts and frozen in liquid nitrogen–cooled freon and stored under liquid nitrogen. The samples were processed in a Balzers BAF400T freeze-fracture apparatus (Balzers Corporation, Nashua, NH) at a stage temperature of −100°C. After fracturing, the specimens were shadowed with platinum-carbon and stabilized by carbon evaporation. The replicas were cleaned in 5% sodium dichromate in 50% H_2SO_4 and retrieved from distilled water on copper grids. Replicas were viewed and photographed with a Zeiss EM-10A transmission electron microscope at an accelerating voltage of 60 kV (*20*).

CF/T43 cells (passages 9 to 19) and NL/T4 cells (passages 5 to 11) developed significant R_t's and equivalent short-circuit currents (I_{eq}) (Table 1). The R_t of the cell lines was lower than that of primary cultures, whereas the transepithelial potential (V_t) and I_{eq} were intermediate between the values reported by different investigators (Table 1). The development of an R_t was accompanied by the formation of morphologic correlates of tight junctions comparable to those of airway epithelial cells in primary culture (Fig. 2). The intracellular Cl^- activity (a_{Cl}) approximated the values of primary cultures.

To assess the basal Cl^- conductance (G_{Cl}) of the apical membrane, we treated CMS cultures with amiloride (10^{-4}*M*) to inhibit apical Na^+ conductance (*11*) and reduced the luminal solution Cl^- concentration to 3

Table 1. Comparison of baseline electrophysiologic properties of CF and normal airway epithelial cells in primary culture with those of CF/T43 and NL/T4 cell lines. Baseline electrophysiologic properties (mean ± SEM) of cultured normal and CF airway epithelial cells. V_t, transepithelial potential difference; V_a, apical membrane potential difference; V_b, basolateral membrane potential difference; fR_a, fractional resistance of the apical cell membrane; R_t, transepithelial resistance; I_{eq}, equivalent short-circuit current; a_{Cl}, intracellular Cl^- activity. Voltages were measured with conventional and ion-specific microelectrodes, and other parameters were calculated as described (*9*).

Source	*n*	V_t (mV)	V_a (mV)	V_b (mV)	fR_a	R_t (ohm cm^2)	I_{eq} (μA cm^{-2})	a_{Cl} (m*M*)
				Cystic fibrosis				
Primary cultures								
Willumsen *et al.* (*9*)	28	−29.2 ± 4.4	−15.6 ± 3.1	−44.8 ± 2.2	0.54 ± 0.03	435 ± 42	76.2 ± 12.9	46.5 ± 2.5
Widdicombe (*16*)	4	−0.6				300 ± 40	1.9 ± 0.6	
Cell line								
CF/T43	18	−2.3 ± 0.3	−25.4 ± 1.9	−27.7 ± 2.0	0.56 ± 0.04	125 ± 12	19.2 ± 2.1	42.4 ± 2.9
				Normal				
Primary cultures								
Willumsen *et al.* (*9*)	34	−10.1 ± 1.1	−26.1 ± 1.2	−36.2 ± 1.2	0.46 ± 0.02	400 ± 35	28.2 ± 3.0	42.7 ± 2.0
Widdicombe (*16*)	6	−1.0				367 ± 38	2.6 ± 0.6	
Cell line								
NL/T4	8	−0.9 ± 0.1	−24.7 ± 1.8	−25.5 ± 1.8	0.67 ± 0.05	79 ± 12	12.8 ± 3.0	34.9 ± 3.6

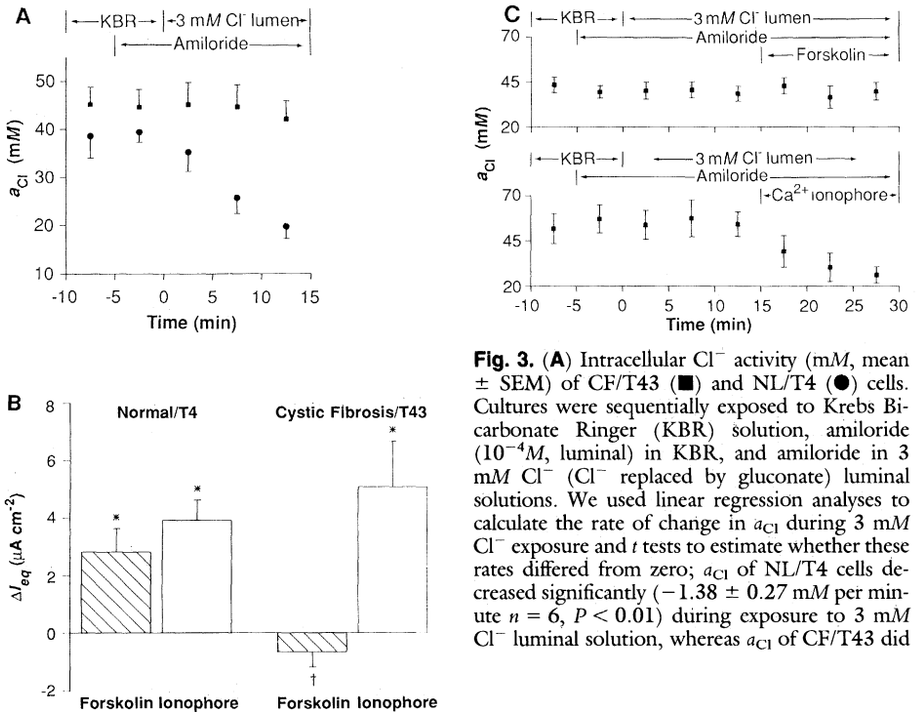

Fig. 3. (**A**) Intracellular Cl^- activity (m*M*, mean ± SEM) of CF/T43 (■) and NL/T4 (●) cells. Cultures were sequentially exposed to Krebs Bicarbonate Ringer (KBR) solution, amiloride (10^{-4}*M*, luminal) in KBR, and amiloride in 3 m*M* Cl^- (Cl^- replaced by gluconate) luminal solutions. We used linear regression analyses to calculate the rate of change in a_{Cl} during 3 m*M* Cl^- exposure and *t* tests to estimate whether these rates differed from zero; a_{Cl} of NL/T4 cells decreased significantly (−1.38 ± 0.27 m*M* per minute $n = 6$, $P < 0.01$) during exposure to 3 m*M* Cl^- luminal solution, whereas a_{Cl} of CF/T43 did not (−0.17 ± 0.09 m*M* per minute, $n = 13$). Intracellular Cl^- activity was measured with ion-selective microelectrodes (*9*). Three to ten impalements for each condition were averaged for each of 13 (CF/T43) or 6 (NL/T4) cultures. (**B**) Change in I_{eq} (mean ± SEM) of amiloride-treated (10^{-4}*M*, apical) NL/T4 and CF/T43 cells exposed to forskolin (10^{-5}*M*, luminal) ($n = 4$) or a Ca^{2+} ionophore [A23187 (10^{-6}*M*) or ionomycin (10^{-5}) luminal] ($n = 4$). I_{eq}'s before secretagogue exposure were 4.0 ± 1.2 and 3.3 ± 1.0 μA cm^{-2} for NL/T4, 6.1 ± 1.1 and 5.5 ± 1.2 μA cm^{-2} for CF/T43. *Different from baseline, $P < 0.05$. †Different from NL/T4 response to forskolin, $P < 0.02$; $P < 0.04$ with Bonferroni correction for multiple comparisons. (**C**) Intracellular Cl^- activity (mean ± SEM) of CF/T43 cells. Cultures were sequentially exposed to KBR, amiloride in KBR, and amiloride in 3 m*M* Cl^- luminal solutions, then exposed to forskolin (10^{-5}*M*, luminal) or a Ca^{2+} ionophore [A23187 (10^{-6}*M*) or ionomycin (10^{-5}*M*) luminal] in amiloride, 3 m*M* Cl^- solution. Intracellular Cl^- activity did not change significantly (−0.04 ± 0.31 m*M* per minute, $n = 6$) during forskolin exposure, but decreased significantly (−1.85 ± 0.26 m*M* per minute, $n = 4$, $P < 0.01$) during ionophore exposure.

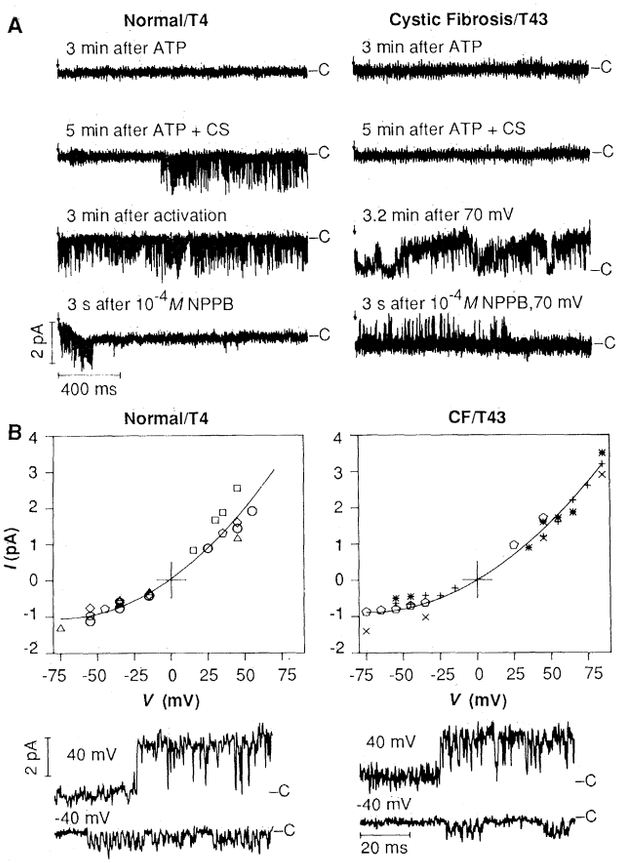

Fig. 4. (**A**) Representative records showing activation of NL/T4 Cl^- channels by ATP plus catalytic subunit (CS) of cAMP-dependent kinase (*21*). CF/T43 Cl^- channels are not activated by ATP plus CS, but are activated by depolarization to +70 mV. Both NL/T4 and CF/T43 Cl^- channels are inactivated by the Cl^- channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) (*22*). Arrows denote the times indicated above each trace. The clamp potential was −40 mV with respect to the pipette, except where noted. C, closed state. Excised inside-out patches were formed by the method of Hamill *et al.* (*23*). The pipette solution was 140 m*M* NaCl, 2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 5 m*M* *N*-tris[hydroxymethyl] methyl-2-aminoethane sulfonic acid (TES), *p*H 7.2. The bathing solution contained 140 m*M* NaCl, 4 m*M* $MgCl_2$, 1 m*M* EGTA, 0.71 m*M* $CaCl_2$, and 5 m*M* TES (*p*H 7.2). The cytoplasmic face of the membrane was first exposed to bathing solution containing 1 m*M* ATP. After 5 min, the bathing solution was changed to one that contained 1 m*M* ATP and 100 n*M* CS. If no activation occurred after 8 to 10 min, the patch voltage was depolarized to 70 to 100 mV. After activation, channels were identified by conductance, kinetics, reversal potential in asymmetric pipette:bathing solutions, outward rectification (*14*), and sensitivity to NPPB. All patch-clamp experiments were performed at 20° to 22°C. (**B**) Top: Current-voltage (*I-V*) relation of Cl^- channels from NL/T4 and CF/T43 cells. Each symbol represents data from a different preparation. Conductance of individual experiments was calculated as the slope of the tangent of the *I-V* relation at 0 mV. Bottom: Traces are representative records demonstrating increased conductance at the +40-mV clamp potential.

m*M* to create a large chemical gradient for Cl^- exit. This led to a reduction of a_{Cl} in NL/T4 but not in CF/T43 cells (Fig. 3A), mimicking the behavior of primary cultures (*9, 10*) and indicating a negligible apical membrane G_{Cl} in CF/T43 cells.

Several approaches were used to evaluate the regulation of apical membrane G_{Cl} in these cell lines. First, the capacity of an adenosine 3′,5′-monophosphate (cAMP)–dependent agonist (forskolin) and Ca^{2+} ionophores to increase the I_{eq} of amiloride-treated cells [an index of net Cl^- secretion (*12*)] was compared in NL/T4 and CF/T43 cells (Fig. 3B). As in freshly excised tissues and primary cultures, forskolin was an effective Cl^- secretagogue in NL/T4 but not in CF/T43 cells, whereas Ca^{2+} ionophores were effective in both cell types. The lack of CF/T43 response to forskolin was not caused by abnormal cAMP accumulation because forskolin increased cAMP levels (*13*) comparably in CF/T43 (from 0.75 ± 0.02 to 7.8 ± 3.0 pmol per milligram of protein, $n = 3$) and NL/T4 (from 1.0 ± 0.3 to 6.1 ± 0.9 pmol per milligram of protein, $n = 3$) cells.

Second, to further assess whether Ca^{2+} ionophores increased apical G_{Cl} in CF/T43 cells, we compared the effects of ionomycin ($n = 1$) and A23187 ($n = 3$) with forskolin in amiloride-treated cultures that were exposed to reduced (3 m*M*) luminal Cl^-. Calcium ionophores, but not forskolin, induced a decrease in a_{Cl} (Fig. 3C), reflecting a Ca^{2+}-mediated activation of an apical membrane G_{Cl}.

Third, we assessed Cl^- channel regulation at the single channel level. Schoumacher *et*

al. and Li *et al.* (*14*) found that the catalytic subunit (CS) of cAMP-dependent kinase activated outwardly rectifying Cl^- channels in excised patches of normal but not CF airway epithelial cells. In 18 inside-out patches from NL/T4 cells, 6 were found to have Cl^- channels (Fig. 4A). Five of these six were activated by 1 m*M* adenosine triphosphate (ATP) and 100 n*M* CS, but not by ATP alone. Chloride channels in the remaining patch were elicited by depolarizing voltage. Of 16 inside-out patches taken from CF/T43 cells, 5 were ultimately found to contain Cl^- channels. None of these were activated by the ATP or ATP plus CS; all were activated by depolarizing voltages. Chloride channels from CF/T43 and NL/T4 had conductances [28.7 ± 1.3 pS (n = 5) and 29.6 ± 2.0 pS (n= 5), respectively] (Fig. 4B) similar to those reported for CF and normal airway epithelial cells in primary culture (*14*).

The ultimate proliferation potential of the CF/T43 cell line remains to be determined. Nevertheless, the demonstrated proliferation capability is adequate to support many experiments. For example, a single cryopreserved vial of 10^6 cells at passage 9 may be expanded to 10^{12} cells at passage 19 (the highest passage currently known to manifest the CF regulatory phenotype). This exceeds by three orders of magnitude the number of CF cells in primary culture produced in 1 year by a large tissue culture core facility (*15*).

We have developed a human airway epithelial cell line that maintains specific differentiated properties and is therefore useful for studies of the biologic properties underlying CF. The extended proliferation capability of the CF/T43 cell line is certainly caused by the SV40T gene, since the T antigen is expressed and control cultures died within four passages. The retention of differentiated epithelial characteristics is probably due to the random site of gene insertion, which determines the expression of T antigen and its effects on cell proliferation and function. Our selection strategy, which focused on the ability of cells to form resistive barriers, may favor clones in which other differentiated cell functions are relatively preserved. The key features of the CF/T43 line—formation of functional tight junctions, reduced apical membrane G_{Cl}, and activation of apical Cl^- channels by Ca^{2+} ionophores but not by cAMP-dependent agonists—suggest that the molecular processes underlying CF are preserved. These cells will be instrumental in elucidating these mechanisms, in testing candidate complementary genes for correction of the observed CF abnormalities, and for developing and testing new therapeutic strategies.

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Regulatory Role for GTP-Binding Proteins in Endocytosis

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Guanosine 5′-triphosphate (GTP)–binding proteins have been implicated in the transport of newly synthesized proteins along the secretory pathway of yeast and mammalian cells. Early vesicle fusion events that follow receptor-mediated endocytosis as measured by three in vitro assays were blocked by guanosine 5′-*O*-(3-thiotriphosphate) and aluminum fluoride. The effect was specific for guanosine nucleotides and depended on the presence of cytosolic factors. Thus, GTP-binding proteins may also have a role in the transport of molecules along the endocytic pathway.

GUANOSINE 5′-TRIPHOSPHATE (GTP)–binding proteins may participate in various intracellular processes in addition to their well-characterized role in the transduction of hormonal and sensory signals (*1*). Analogs of GTP have been shown to affect exocytosis in permeabilized cells (*2*), and studies of yeast mutants defective in the secretory pathway have identified GTP-binding proteins that are required for normal secretion (*3*). These proteins have extensive homology with *ras* gene products (*4*). Ras proteins are GTP-binding proteins that appear to participate in the proliferation of eukaryotic cells (*5*). In mammalian cells, evidence for a regulatory mechanism mediated by GTP-binding proteins in the secretory pathway comes from the observation that transport from the endoplasmic reticulum to the Golgi apparatus (*6*) and among Golgi stacks (*7*) is inhibited by guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) (a nonhydrolyzable GTP analog) in cell-free systems. In addition, GTP-γ-S affects the in vitro recycling of phosphomannosyl receptors to the Golgi apparatus (*8*).

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