Demonstration that CFTR is a Chloride Channel by Alteration of its Anion Selectivity
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11. One example of an experiment employing target cells from 129 inbred mice can be seen in Fig. 3, experiment 1. The 129 inbred line was initiated by breeding the original mosaic founder animal (*6*) with 129/Sv mice, and interbreeding the +/− offspring; since the embryonic stem cell line is of 129/Sv origin, this procedure yields inbred 129 −/− animals.
12. M. Zijlstra, unpublished data showing restoration of class I expression by the human β_2M transgene (*35*).
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30. To activate NK cells in vivo, mice were inoculated intraperitoneally one day before an experiment with poly I:C (100 μg) (*9, 10*). To enrich NK cells, spleen cells were depleted of non-NK cells bearing various markers with the use of MAbs plus complement [a mixture of guinea pig and rabbit sera (*36*)]. MAbs to IA^b (BP107), heat stable antigen (J11d), CD4 (GK1.5), and CD8 [AD4(15)] were used as indicated. After the depletions, viable cells were isolated on a Ficoll gradient.
31. The cytotoxic cell assay was as described (*36*) except that medium containing a serum substitute (AIM V medium, Gibco, Gaithersburg, MD) was employed for preparing the Con A blast target cells and in the assay cultures. However, when the assay was performed with serum or with AIM V medium on the same day, no difference was observed in the lysis of −/− cells by enriched NK cells.
32. Spleen cells from B6 mice pretreated with poly I:C were first depleted of B cells by treating the cells with anti-IA^b plus complement (*30*) and isolating the surviving cells on a Ficoll gradient. Aliquots of these cells were treated with antibodies plus complement (*36*). Following the complement treatment, viable cells were isolated on a Ficoll gradient, washed, and tested for lysis of YAC-1 tumor cells, or −/− or +/− Con A blast targets from (B6 × 129)F_2 and F_3 mice as indicated. Cell numbers were not adjusted for the numbers of cells killed by antibody and complement, so the effector/target ratio in these panels refers to input effector cell number.
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34. The staining experiment in Fig. 4 employed third-generation backcross mice of (B6 × 129)F_1 +/− mice to B6 mice; it was necessary to use B6 backcross mice, because the B6 strain but not the 129 strain expresses the NK1.1 allele. Viable nucleated spleen cells from the indicated mice were isolated on a Ficoll gradient, incubated with biotinylated MAbs (PK136) to NK1.1 (*33*), washed, and stained with phycoerythrin-streptavidin (Southern Biotechnology, Birmingham, AL) and fluorescein-conjugated MAb H57-597, specific for TCRβ antibody (*37*). The stained cells were analyzed on an Epics C flow cytometer and the data presented on a logarithmic scale. Dead cells were excluded by their forward and right angle light scattering characteristics.
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Demonstration That CFTR Is a Chloride Channel by Alteration of Its Anion Selectivity

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Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) generates adenosine 3′,5′-monophosphate (cAMP)–regulated chloride channels, indicating that CFTR is either a chloride channel or a chloride channel regulator. To distinguish between these possibilities, basic amino acids in the putative transmembrane domains were mutated. The sequence of anion selectivity of cAMP-regulated channels in cells containing either endogenous or recombinant CFTR was bromide > chloride > iodide > fluoride. Mutation of the lysines at positions 95 or 335 to acidic amino acids converted the selectivity sequence to iodide > bromide > chloride > fluoride. These data indicate that CFTR is a cAMP-regulated chloride channel and that lysines 95 and 335 determine anion selectivity.

CYSTIC FIBROSIS (CF) (*1*) IS CAUSED by mutations in the gene encoding CFTR (*2–6*). Amino acid sequence analysis and comparison with other proteins (*3, 7, 8*) suggest that CFTR consists of two repeats of a unit containing six membrane-spanning segments and a putative nucleotide binding domain (Fig. 1). The two repeats are separated by a large polar segment called the R (regulatory) domain, which contains multiple potential phosphorylation sites. The predicted topology of CFTR, with the exception of the R domain, resembles that of a number of other membrane proteins, such as the multiple drug resistance P-glycoprotein, the yeast STE6 gene product, and several bacterial periplasmic permeases (*3, 7, 8*).

Chloride transport by epithelial tissues is abnormal in patients with CF (*9*); apical membrane Cl^- channels do not open in response to an increase in intracellular cAMP. When CFTR is expressed in cell types that do not normally express CFTR or have cAMP-regulated Cl^- channels (*10, 11*), cAMP-regulated Cl^- currents are generated. The simplest interpretation of these results is that CFTR is a cAMP-regulated Cl^- channel, but it is possible that CFTR could regulate endogenous, previously silent Cl^- channels. The notion that CFTR is a Cl^- channel has been controversial because CFTR does not resemble any known ion channels but most resembles a family of energy-dependent transport proteins (*3, 7, 8*). To test whether CFTR is a cAMP-regulated Cl^- channel, we used site-directed mutagenesis to change the properties of CFTR.

As permeating ions flow through an ion-selective pore, they sense the electrostatic forces generated by amino acids that line the pore. This interaction between amino acids and the permeating ions determines ionic selectivity (*12*). We reasoned that if CFTR is a Cl^- channel, then changing positively charged amino acids in CFTR to negatively charged amino acids might alter ionic selectivity. Similar strategies have been used in K^+ channels, the nicotinic acetylcholine receptor, and the mitochondrial voltage-dependent anion-selective channel (*13*).

We mutated amino acids within the putative membrane-spanning sequences (M1 through M12) (Fig. 1) identified by Riordan and co-workers (*3*). On the basis of their hydropathy and the prediction that they form α helices, these sequences are

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likely to span the lipid bilayer. They are also highly conserved among different species (*14*), suggesting conservation of function. Moreover, a mutation that changes Arg^{347} in M6 to proline may be responsible for some cases of CF (*15*), indicating that this residue may be critical for CFTR function. We therefore made the following mutations, converting basic residues to acidic residues: $Lys^{95} \rightarrow Asp^{95}$, $Lys^{335} \rightarrow Glu^{335}$, $Arg^{347} \rightarrow Glu^{347}$, and $Arg^{1030} \rightarrow Glu^{1030}$ (*16*).

In HeLa cells transfected with either the wild type or the mutant forms of CFTR, cAMP reversibly activated whole-cell currents (Table 1). As reported with the vaccinia virus–T7 hybrid expression system (*5, 10, 17*), 30 to 70% of the cells responded to cAMP. We compared the properties of cAMP-regulated current in cells expressing wild type and mutant CFTR with cAMP-regulated current in the apical membrane of T84 epithelial cells (which normally express CFTR) (*18*).

Representative current responses of CFTR to hyperpolarizing and depolarizing voltage steps are shown in Fig. 2. Wild-type CFTR, mutated CFTR, and Cl^- channels in the apical membrane of T84 cells showed similar responses: the majority of the current was voltage-insensitive.

In symmetrical Cl^- concentrations, CFTR generates Cl^- channels that display a linear current-voltage (*I-V*) relation (*10, 11*). To maximize our ability to detect a change in the relative permeability of Na^+ to Cl^-, we used a NaCl concentration gradient (Fig. 3). Under these conditions, rectification (or curvature) of the *I-V* relations probably results from the asymmetric Cl^- concentrations. Similar permeabilities for Cl^- over Na^+ were observed in mutated and nonmutated CFTR and in the apical membrane of T84 cells (Table 1). Our mutations did not cause a general disruption of channel structure because cAMP-dependent channel regulation was intact, voltage-dependence was unchanged, and selectivity for Cl^- over Na^+ was preserved.

The anion permeability sequence of cAMP-regulated currents in wild-type CFTR and the apical membrane of T84 cells was $Br^- \geq Cl^- > I^- > F^-$ (Fig. 3, A and B, and Table 1) (*19*). Site-directed mutation altered the anion selectivity. When Lys^{95} or Lys^{335} were substituted with amino acids containing acidic side chains, the anion permeability sequence was converted to $I^- > Br^- > Cl^- > F^-$ (Fig. 3, C and D, and Table 1). When Arg^{347} and Arg^{1030} were mutated, the permeability sequence was not different from that of wild-type CFTR, although the relative permeability of I^- to Cl^- increased (Fig. 3, E and F, and Table 1).

We also estimated the relative anion conductance of these mutated channels (*20*). Wild-type CFTR, CFTR-R347E, and CFTR-R1030E had cAMP-dependent channels with relative conductance sequences that paralleled their permeability sequences: $Br^- \geq Cl^- > I^- \approx F^-$. The most dramatic change was in CFTR-K335E, for which the conductivity sequence was $Br^- > I^- > Cl^- \geq F^-$ (Fig. 3D). Although CFTR-K95D showed an altered permeability sequence, the relative conductivity sequence for the halides remained unchanged from that for the wild-type channel.

Eisenman's equilibrium theory of ionic selectivity (*21*) explains why removing positive charge might alter the anion selectivity from $Cl^- > I^-$ to $I^- > Cl^-$. According to Eisenman's theory, anionic selectivity depends on the hydration energy of the anion and the energy of interaction between an anion and a positively charged site (*22*). When the anion-site interaction energy is less than the energy required for dehydration, the permeability sequence is $I^- > Br^- > Cl^- > F^-$, but when the anion-site interaction energy is much greater than the energy required for dehydration, the permeability sequence is $F^- > Cl^- > Br^- > I^-$. The permeability sequence of CFTR ($Br^- > Cl^- > I^- > F^-$) suggests that CFTR con-

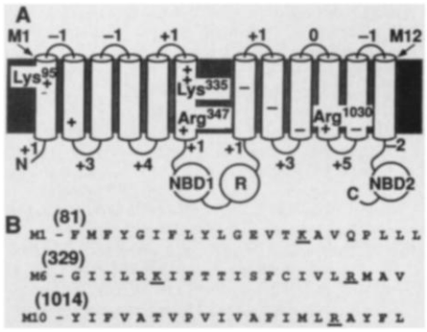

Fig. 1. Predicted topology of CFTR in the membrane. (**A**) M1 to M12, membrane-spanning sequences; NBD1 and NBD2, nucleotide-binding domains; R, regulatory domain. Charged residues in M1 to M12 are indicated by + and −. Predicted net charges of intracellular and extracellular loops are indicated; charge of first five amino acids only, before M1 and M7 and after M6 and M12, are indicated. (**B**) Sequences of M1, M6, and M10. Amino acids mutated in this study are underlined. The single-letter code for the amino acids is as follows: A, Ala; R, Arg; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; Y, Tyr; V, Val.

Table 1. Relative anion permeability and conductance of cAMP-stimulated channels in the apical membrane and in cells expressing wild-type (*27*) and mutant CFTR. Data are mean ±SEM of values calculated from currents in cAMP-stimulated individual cells (*n*, number of cells). Solutions and cAMP stimulation are as in Fig. 2. During stimulation, the mucosal or extracellular (bath) solution was changed from a Cl^- solution to one containing each of the indicated anions. To check for reversibility, cAMP was removed. Permeability ratios P_X/P_{Cl}, where X is Na^+, I^-, Br^-, or F^-, were calculated from reversal potentials with the Goldman-Hodgkin-Katz equation. Chord conductance was measured as the slope between the reversal potential and the reversal potential plus 25 mV. Conductance ratios G_X/G_{Cl} are the ratios of I^-, Br^-, and F^- to Cl^-. Liquid junction potentials and potentials at the tip of the patch pipette were corrected according to E. M. Fenwick *et al.* (*28*). The chord conductance of cAMP-regulated currents across the apical membrane was 1.67 ± 0.12 mS cm^{-2} (n = 12). The chord conductance of whole-cell currents (in nS) before/after stimulation with a NaCl bath solution was (number of experiments in parentheses) as follows CFTR-3T3, 2.6 ± 0.8/51.7 ± 20.2 (4); CFTR, 0.8 ± 0.6/22.4 ± 17.4 (5); K95D, 0.3 ± 0.1/3.5 ± 1.3 (4); K335E, 0.5 ± 0.2/6.5 ± 2.2 (5); R347E, 1.0 ± 0.6/11.5 ± 3.2 (4); R1030E, 0.5 ± 0.3/27.2 ± 9.9 (4).

Protein (n)	P_{Na}/P_{Cl}	P_X/P_{Cl}				G_X/G_{Cl}			
		Br^-	Cl^-	I^-	F^-	Br^-	Cl^-	I^-	F^-
				T84 epithelia					
Apical (12)	0.16 ±0.01	1.21 0.06	1.00	0.56 ±0.03	0.11 ±0.03	0.92 0.05	1.00	0.47 ±0.03	0.64 ±0.02
				NIH 3T3 cells					
CFTR (4)	0.01 ±0.01	1.11 ±0.03	1.00	0.59 ±0.07	0.30 ±0.01	1.26 ±0.19	1.00	0.29 ±0.04	0.15 ±0.07
				HeLa cells					
CFTR (5)	0.09 ±0.03	1.24 ±0.09	1.00	0.57 ±0.08		1.02 ±0.13	1.00	0.39 ±0.04	
K95D (4)	0.09 ±0.02	1.25 ±0.12	1.00	1.43 ±0.05	0.15 ±0.05	1.39 ±0.32	1.00	0.75 ±0.05	0.48 ±0.18
K335E (5)	0.11 ±0.02	1.06 ±0.02	1.00	1.37 ±0.09	0.15 ±0.00	1.71 ±0.28	1.00	1.43 ±0.23	0.69 ±0.21
R347E (4)	0.10 ±0.01	1.24 ±0.06	1.00	0.90 ±0.08	0.13 ±0.02	1.46 ±0.27	1.00	0.47 ±0.16	0.51 ±0.28
R1030E (4)	0.12 ±0.01	1.46 ±0.07	1.00	0.81 ±0.10	0.33 ±0.05	1.50 ±0.12	1.00	0.28 ±0.03	0.52 ±0.10

tains a pore with a moderately high affinity site for anions. Because replacement of the positively charged amino acids Lys^{95} or Lys^{335} with negatively charged amino acids changed the selectivity sequence of CFTR to that of a low affinity site ($I^- > Br^- > Cl^- > F^-$), the data suggest that these amino acids contribute to such a site.

The orientation of CFTR shown in Fig. 1 is based on hydropathy plots (*3*), the location of glycosylation sites (*23*), the lack of a signal sequence, which suggests a cytoplasmic NH_2-terminus (*3*), and the intracellular location of the R domain and COOH terminus (*24*). In this model, Lys^{95} and Lys^{335} lie in the outer half of the channel; mutation of either has similar dramatic effects on anion permeability. Residues Arg^{347} and Arg^{1030} lie in the inner half of the channel; mutation of either has only minor effects on anion permeability.

Our demonstration that CFTR forms a cAMP-regulated Cl^- channel indicates that CFTR has a function not described for other members of the family of energy-dependent transport proteins (*3, 7, 8*). What then is the function of the two nucleotide-binding domains found in CFTR? Although CFTR forms a pore that passively conducts Cl^-, our data do not exclude the possibility that CFTR could have an additional function; for example, it might also actively transport some other unidentified substrate. Alternatively, the nucleotide-binding domains could serve a regulatory function rather than drive a pump.

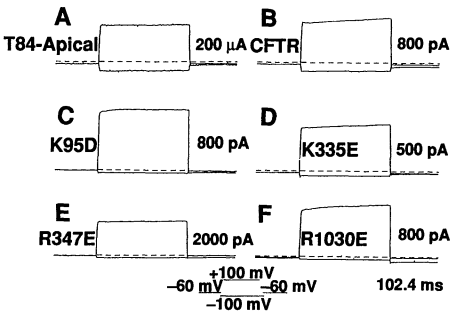

Fig. 2. Current response to hyperpolarizing and depolarizing voltage steps in (**A**) the apical membrane of T84 intestinal epithelia (T84) and in (**B**) HeLa cells expressing wild-type CFTR or mutant [(**C**) K95D, (**D**) K335E, (**E**) R347E, or (**F**) R1030E] CFTR (*29*). Data represent examples of cAMP-stimulated current at voltage steps to ±100 mV from a holding voltage of −60 mV in stimulated cells. We increased cAMP with either 10 μM forskolin plus 100 μM 3-isobutyl-1-methylxanthine or 500 μM CPT-cAMP [8-(4-chlorophenylthio) adenosine cyclic monophosphate] (both gave similar results). In T84 cells, cAMP was increased by adding the sodium salt of cAMP (20 μM) to the serosal solution. We measured cAMP-stimulated currents in the apical membrane by permeabilizing the basolateral membrane (*29, 30*) and in cells expressing CFTR by the standard (*31*) whole-cell voltage-clamp technique (*10*). Cultures of T84 intestinal epithelia were grown and studied as described (*29*). Voltage is referenced to the bath in whole-cell studies and to the mucosal solution in apical membrane studies; positive current (upward deflection) represents the flow of anions into the cell or from mucosal solution into the cell. Intracellular and serosal solutions contained 120 mM *N*-methyl-D-glucamine, 115 mM aspartic acid, 3 mM $MgCl_2$, 1 mM Na_2ATP, and 5 mM Hepes (pH 7.3 with 7 mM HCl). Cesium EGTA (1 mM) was added in whole-cell studies. Extracellular and mucosal solutions contained 140 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM dextrose, and 10 mM Hepes (pH 7.3 with 4.5 mM NaOH). For T84 cells, baseline current was subtracted. Dashed line, the zero current level. Series resistance was compensated as described (*10*). Studies were performed at 30 to 35°C.

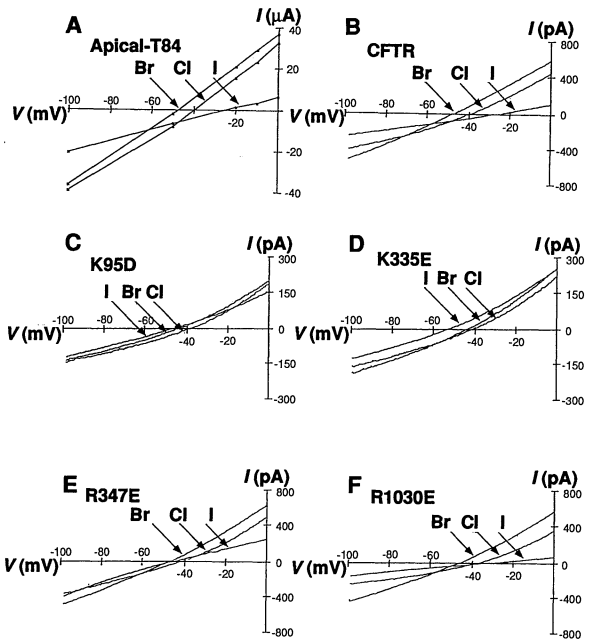

Fig. 3. *I-V* relations of cAMP-stimulated currents in (**A**) the apical membrane of T84 intestinal epithelial (T84) and in HeLa cells expressing (**B**) wild-type CFTR or mutant [(**C**) K95D, (**D**) K335E, (**E**) R347E, or (**F**) R1030E] CFTR. Data represent examples from single cells. Methods and solutions are as in Fig. 2. Extracellular and mucosal solutions contained either 140 mM NaCl, 140 mM NaBr, or 140 mM NaI. *I-V* relation of apical membrane Cl^- current of T84 cells (A) was obtained by subtracting baseline values from stimulated values. *I-V* relations of wild-type and mutant CFTR were obtained from voltage ramps from −100 mV to 0 mV over a 1-s period. Baseline currents were not significantly different for wild-type or mutant CFTR. Data shown are not corrected for liquid junction potentials or potentials at the tip of the patch pipette.

Our data indicate that CFTR is a cAMP-regulated Cl^- channel. The finding that apical membrane Cl^- channels and Cl^- channels induced by recombinant CFTR share several properties is consistent with that conclusion (*25*). The fact that specific mutations in the membrane spanning sequences alter anion selectivity demonstrates that CFTR forms a cAMP-regulated anion pore and makes other interpretations unlikely. This conclusion begins to explain how genetic mutations in the gene for CFTR cause the pathophysiology of the disease (*1, 9*).

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25. The properties of CFTR are different from those of the outwardly rectifying Cl^- channel [M. J. Welsh, *FASEB J.* **4**, 2718 (1990)]: that channel has a rectifying *I-V* relation, whereas CFTR has a linear relation (*6, 10, 11*); that channel is more permeable to I^- than to Cl^-, whereas CFTR is more permeable to Cl^- than to I^- (Table 1); and that channel is inhibited by extracellular DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid) and IAA-94, whereas CFTR is not (M. P. Anderson and M. J. Welsh, unpublished data). It is not clear how mutations in CFTR produce defective regulation of outwardly rectifying Cl^- channels in CF epithelia.
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Effect of Deleting the R Domain on CFTR-Generated Chloride Channels

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The cystic fibrosis transmembrane conductance regulator (CFTR), which forms adenosine 3′,5′-monophosphate (cAMP)–regulated chloride channels, is defective in patients with cystic fibrosis. This protein contains two putative nucleotide binding domains (NBD1 and NBD2) and an R domain. CFTR in which the R domain was deleted (CFTRΔR) conducted chloride independently of the presence of cAMP. However, sites within CFTR other than those deleted also respond to cAMP, because the chloride current of CFTRΔR increased further in response to cAMP stimulation. In addition, deletion of the R domain suppressed the inactivating effect of a mutation in NBD2 (but not NBD1), a result which suggests that NBD2 interacts with the channel through the R domain.

CYSTIC FIBROSIS (CF) (*1*) IS CAUSED by mutations in CFTR (*2–5*), which generates cAMP-regulated Cl^- channels (*6–8*). The primary amino acid sequence of CFTR predicts that the protein has two repeated units, each containing a membrane-spanning domain and a nucleotide binding domain (NBD), separated by a unique segment named the R domain (*3*). The R domain has a number of potential phosphorylation sites for cAMP-dependent protein kinase (*3*). In addition, CFTR can be phosphorylated by cAMP-dependent protein kinase (*9*). We therefore tested whether the R domain confers cAMP dependence on the CFTR Cl^- channel.

To address this question, we examined the consequences of deleting the R domain (*10*) (Fig. 1). We constructed a plasmid-encoding CFTR in which amino acids 708 to 835 were deleted (CFTRΔR) (*11*), expressed it in HeLa cells (*12*) (Fig. 1B), and assessed cAMP-dependent Cl^- channel activity with the halide-sensitive fluorophore 6-methoxy-*N*-(3-sulfopropyl)-quinolinium (SPQ) (*13*). In the SPQ assay, an increase in halide permeability results in a more rapid increase in SPQ fluorescence (*4, 6*).

Substitution of NO_3^- for I^- in cells expressing CFTR produced minimal changes in SPQ fluorescence (Fig. 1A). A subsequent increase in intracellular cAMP, produced by addition of forskolin and 3-isobutyl-1-methylxanthine (IBMX), stimulated a rapid increase in fluorescence, indicating that cAMP increased anion permeability (*4, 6*). In contrast, in unstimulated cells expressing CFTRΔR, substitution of I^- by NO_3^- caused an immediate, rapid increase in SPQ fluorescence (Fig. 1A), a response that resembled that observed in CFTR-expressing cells stimulated by cAMP. Subsequent increase of cAMP concentrations by forskolin and IBMX in cells expressing CFTRΔR further increased the rate of change in SPQ fluorescence.

Cells expressing CFTRΔR had large basal currents as measured by the whole-cell patch-clamp method, even in the absence of cAMP (Fig. 2, A and B). In contrast, increased cAMP concentrations were required to stimulate Cl^- currents in cells expressing CFTR (*4, 6,* 7) (Fig. 1). Such currents were not present in nontransfected HeLa cells (basal current, 15 ± 3 pA; n = 8) or CFTR-transfected cells (basal current, 93 ± 48 pA; n = 8) (*6*). Stimulation that raised cAMP concentrations produced a further increase in whole-cell Cl^- current in cells expressing CFTRΔR (Fig. 2, A and B); basal current was 1041 ± 204 pA (n = 7) at +80 mV and increased by 32 ± 11% upon stimulation with cAMP. Activation was reversible in six of six cases.

Currents seen after expression of CFTRΔR in unstimulated cells were similar to CFTR-generated currents in cAMP-stimulated cells (*4, 6,* 7): both currents were selective for Cl^- (Fig. 2, C and D, and Table

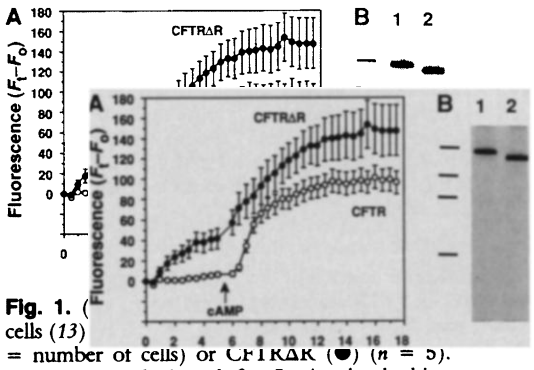

Fig. 1. (cells (*13*) = number of cells) or CFTRΔR (●) (n = 5). NO_3^- was substituted for I^- in the bathing medium at 0 min. Five minutes later (arrow) cells were stimulated with 20 μM forskolin and 100 μM IBMX (cAMP). Data are mean ± SEM. Without addition of forskolin and IBMX the fluorescence did not increase further after 5 min in either group. (**B**) Expression of CFTR and CFTRΔR in transfected HeLa cells. Cells were transfected with pTM-CFTR4 (lane 1) or pTM-CFTR4ΔR (lane 2) (*12*). Twelve hours after transfection, cells were incubated with [^{35}S]methionine (25 μCi/ml) for 1 hour and lysed in 50 mM tris (pH 7.5), 150 mM NaCl, aprotinin (100 μg/ml), 0.1 mM phenylmethylsulfonyl fluoride, and 1% digitonin (250 μl per 35-mm dish). CFTR and CFTRΔR were immunoprecipitated from cell lysates with a monoclonal antibody to a synthetic peptide from the COOH-terminus of CFTR (amino acids 1466 to 1480) (*26*). Immunoprecipitates (50 μl per lane) were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. Molecular weight standards are 170, 94, 67, and 43 kD.

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