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27. NIH 3T3 fibroblasts were infected with retrovirus expressing human CFTR. 4.5 kb of the wild-type CFTR cDNA (from the initiating ATG to the Sac I site at nucleotide position 4622) was subcloned into a retroviral vector (S. Thompson and R. C. Mulligan, manuscript in preparation). The resultant plasmid was cotransfected with pSV2 *neo* [P. J. Southern and P. J. Berg, *Mol. Appl. Genet.* **1**, 327 (1982)] into CRE cells [O. Danos and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6460 (1988)]. Forty-eight hours after transfection, selection was applied (G418, 1 mg/ml). Medium harvested from G418-resistant cells was used to infect NIH 3T3 fibroblasts with a single 4-hour exposure. The efficiency of infection was determined by Southern (DNA) analysis: approximately 80% of the NIH 3T3 cell population contained an intact proviral genome.
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30. Addition of *Staphylococcus aureus* alpha toxin [M. R. Carson, S. S. Shasby, D. M. Shasby, *Am. J. Physiol.* **257**, L259 (1989)] to the submucosal surface of T84 epithelia permeabilizes the basolateral membrane to monovalent and divalent ions but leaves the apical membrane intact (L. Ostedgaard, D. M. Shasby, M. J. Welsh, manuscript in preparation).
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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

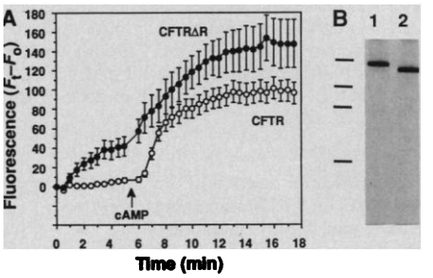

Fig. 1. (**A**) Fluorescence of SPQ-loaded HeLa cells (*13*) expressing CFTR (○) (n = 17, where n = number of cells) or CFTRΔR (●) (n = 5). NO_3^- was substituted for I^- in the bathing medium at 0 min. Five minutes later (arrow) cells were stimulated with 20 μM forskolin and 100 μM IBMX (cAMP). Data 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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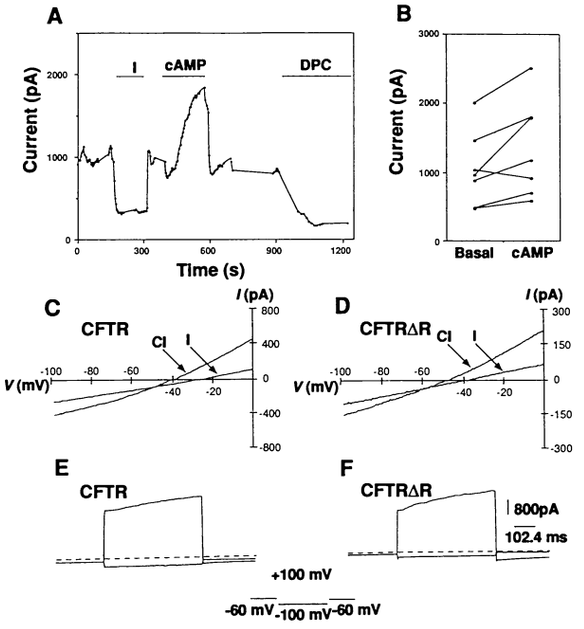

Fig. 2. Whole-cell patch-clamp studies of CFTRΔR- and CFTR-transfected HeLa cells. (**A**) Time course of current for CFTRΔR when extracellular Cl^- was replaced with I^- (I), and 10 μM forskolin and 100 μM IBMX (cAMP) or 0.5 mM DPC were added. Cl^- is present throughout, except where the bar is labeled I^-. Circles indicate data points. Current in (A) and (B) was measured during steps to +80 mV from a holding potential of −60 mV. (**B**) Current measured in seven cells expressing CFTRΔR before (Basal) and during (cAMP) stimulation. Current increased upon addition of either 10 μM forskolin and 100 μM IBMX (n = 3) or 500 μM 8-(4-chlorophenylthio) adenosine cyclic monophosphate (CPT-cAMP) (n = 3). (**C** and **D**) Current-voltage (I-V) relations from a cell expressing (C) CFTR (stimulated by cAMP agents) and a cell expressing (D) CFTRΔR (basal conditions). We used voltage ramps from −100 mV to 0 mV over 1 s in the presence of extracellular Cl^- or I^-. (**E** and **F**) Examples of CFTR-generated current (stimulated by cAMP agents) and CFTRΔR-generated current (basal conditions) during voltage steps to ±100 mV from a holding voltage of −60 mV. We cannot accurately determine the percent of cells expressing functional CFTRΔR when using the whole-cell patch-clamp technique. Of 33 cells studied, 12 had low currents, (<250 pA at +80 mV), 9 had high currents (>250 pA at +80 mV) not Cl^--selective, and 12 had high currents (>250 pA at +80 mV) Cl^--selective (E_{rev} < −30 mV) or displaying $P_{Cl} > P_I$ and $G_{Cl} > G_I$ or both. The latter cells were studied further. Twelve of 33 cells (36%) expressed CFTRΔR, within the range previously observed (*4, 6, 7*). In cells with high non–Cl^--selective basal currents (probably due to a poor membrane seal), a small Cl^--selective current could have been undetected. Moreover, such Cl^--selective currents were never obtained in nontransfected cells (*4, 6, 7*). Dashed line, zero current level. Currents were measured as described (*7, 27*). The intracellular (pipette) solution contained 120 mM *N*-methyl-D-glucamine, 115 mM aspartic acid, 3 mM $MgCl_2$, 1 mM cesium EGTA, 1 mM Na_2ATP, and 5 mM Hepes (pH 7.3 with 7 mM HCl), and the extracellular solution contained 140 mM NaCl or NaI, 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). Studies were at 30° to 35°C, 9 to 18 hours after transfection, for at least three different transfected cultures.

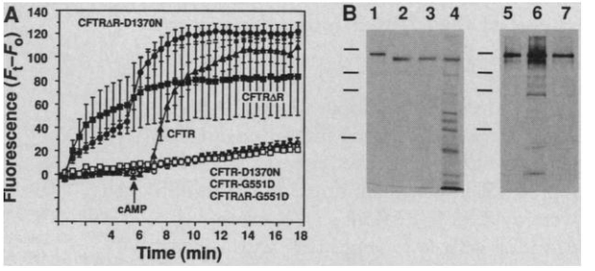

Fig. 3. Effect of deletion of the R domain on CFTR containing NBD2 and NBD1 mutations. (**A**) Fluorescence of HeLa cells expressing CFTR (▲) (n = 18), CFTRΔR (■) (n = 5), CFTRΔR-G551D (△) (n = 21), CFTRΔR-D1370N (●) (n = 14), CFTR-G551D (□) (n = 28), and CFTR-D1370N (○) (n = 15) after substitution of I^- by NO_3^-. Arrow, forskolin (20 mM) and IBMX (100 mM). (**B**) Autoradiograph of CFTR (lane 1), CFTRΔR (lane 2), CFTRΔR-G551D (lane 3), CFTRΔR-D1370N (lane 4), CFTR-D1370N (lane 5), CFTR-G551D (lane 6), and CFTR (lane 7). Samples were prepared as in Fig. 1: samples in lanes 1 to 4 were immunoprecipitated by a monoclonal antibody to the COOH-terminus of CFTR (*26*) and samples in lanes 5 to 7 were immunoprecipitated by a monoclonal antibody to the R domain (*9*). Molecular weight standards as in Fig. 1.

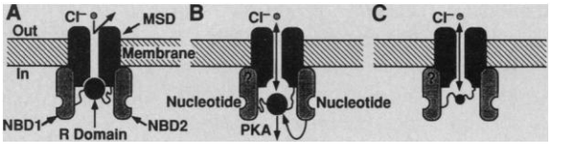

Fig. 4. Model of CFTR. (**A**) Domains of CFTR. MSD, the membrane-spanning domain. (**B**) Phosphorylation of the R domain by cAMP-dependent protein kinase (PKA) opens the channel. A mutation in NBD2 (D1370N) prevents opening through an effect on the R domain. It is not understood how a mutation in NBD1 (for example, G551D) prevents opening (*21*). (**C**) CFTRΔR is open without an increase in cAMP and in the presence of a mutation in NBD2. Although the R domain is shown as a plug that occludes the channel pore, other alternatives are equally feasible.

1) (*7*); both were more permeable and conductive to Cl^- than to I^- (Fig. 2, C and D, and Table 1) (*7*); both were inhibited by diphenylamine-2-carboxylate (DPC) [DPC reduced CFTRΔR currents to an average of 28% (range, 23 to 33%; n = 3) (Fig. 2A), results that were comparable to those for cAMP-stimulated CFTR currents (*4, 7*)]; and most of the current showed time-independent voltage effects (Fig. 2, E and F).

Our data indicate that the R domain (amino acids 708 to 835) confers part of the cAMP dependence on the CFTR Cl^- channel (*14*), because CFTRΔR-generated Cl^- channels were open, even without an increase in cAMP. However, the ability of cAMP to stimulate a further increase in Cl^- current in CFTRΔR suggests that the deleted sites are not the sole regulator of the channel: site or sites sensitive to cAMP-dependent phosphorylation may still be present within CFTRΔR (*15*). Such a site might include potential phosphorylation sites in the portion of CFTR encoded by exon 13 but not deleted in CFTRΔR or sites in another part of the protein (*16*). Because we could delete a large portion of CFTR and yet many functions of the protein were maintained, these deletions did not cause a nonspecific disruption of the entire protein.

CFTR belongs to a family of membrane proteins that includes MDR, yeast STE6, and several bacterial transporters (*3, 17, 18*); NBDs are a dominant, conserved feature of this family. Although the function of the two NBDs in CFTR is unknown, mutations in the NBDs (particularly NBD1) cause CF (*19*). Many mutations in the NBDs lead to defects in glycosylation and potentially in membrane delivery of CFTR (*20*). Other NBD mutations are, however, correctly processed, yet still prevent cAMP-dependent Cl^- channel activation (*21*). Both CFTR-G551D and CFTR-D1370N are processed to a mature, fully glycosylated form; yet both mutant proteins are functionally inactive (Fig. 3) (*20, 21*). Hence, we introduced these mutations into the NBDs of CFTRΔR. CFTR-G551D is associated with CF (*20, 22*).

In cells expressing CFTRΔR-G551D, anion permeability was low in the presence and absence of cAMP (Fig. 3); thus this mutation has the same effect as CFTR-G551D (Fig. 3) (*21*). However, expression of CFTRΔR-D1370N increased both basal and cAMP-stimulated anion permeability, reversing the effects of an NBD2 mutation (CFTR-D1370N) (Fig. 3). This result suggests an interaction between NBD2 and the R domain.

Our data suggest a speculative model for CFTR (Fig. 4). The membrane-spanning

Table 1. Relative anion permeability (P) and conductance (G) of currents generated by CFTR and CFTRΔR. CFTR-generated currents were obtained in cells treated with 10 μM forskolin and 100 μM IBMX; CFTRΔR-generated currents were obtained under basal conditions. Data are mean ± SEM of values from individual cells; n, number of cells. Procedures are as in Fig. 2. Permeability ratios, P_{Na}/P_{Cl} and P_I/P_{Cl}, were determined from reversal potentials, measured from the I-V relations (Fig. 2, C and D) and calculated with the Goldman-Hodgkin-Katz voltage equation (7). Conductance ratios, G_I/G_{Cl}, were determined from the slope between the reversal potential and the reversal potential plus 25 mV. Liquid junction potentials and potentials at the tip of the patch pipette were corrected (*28*).

Expressed protein	n	P_{Na}/P_{Cl}	P_I/P_{Cl}	G_I/G_{Cl}
CFTR	5	0.09 ±0.03	0.57 ±0.08	0.39 ±0.04
CFTRΔR	5	0.13 ±0.04	0.61 ±0.06	0.38 ±0.04

domains form an anion-conducting pore (*6–9*) (Fig. 4). As proposed (*8*), the R domain serves a regulatory role and keeps the channel closed (Fig. 4A): inhibition by the R domain may be released by phosphorylation with cAMP-dependent protein kinase, thereby opening the channel and allowing Cl^- to flow through the pore (Fig. 4B). This model is supported by the observations that CFTR is phosphorylated by cAMP-dependent protein kinase (*9, 23*); the R domain contains multiple potential phosphorylation sites (*3, 23*); an increase in cellular cAMP activates CFTR Cl^- channels (*6*); and expression of CFTRΔR produces a channel that is open even without an increase in intracellular cAMP (Fig. 4C). Phosphorylation might cause a conformational change in the R domain that prevents its blockade of the channel pore. Alternatively, phosphorylation-induced changes in charge might produce electrostatic forces (*24*) that alter the interaction between the R domain and another part of the protein.

The function of the NBDs remains unknown. The finding that mutations in the NBDs prevent channel activation has suggested they might play some regulatory role. Our data suggest that a mutation in NBD2 affects channel activation through an interaction with the R domain. Deletion of most of the R domain suppressed the effect of an NBD2 mutation (D1370N) that inhibits function: not only was basal anion permeability increased, but cAMP further stimulated anion permeability. Because deletion of the R domain did not reverse the effect of a mutation in NBD1, we cannot ascertain whether a similar interaction occurs between NBD1 and the R domain. However, this result suggests that NBD1 could interact with other parts of the protein, for instance, the membrane-spanning domains. Our results with the NBD2 mutation are somewhat analogous to suppressor mutations in prokaryotic members of the family that includes CFTR (*25*).

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10. The R domain is the sequence encoded by exon 13 (*3*). We compared CFTR to the multidrug resistance proteins (MDR) [P. Gros, J. M. Croop, D. E. Houseman, *Cell* **47**, 371 (1986); C. Chen *et al.*, *ibid.*, p. 381], which are similar in topological organization to CFTR but lack a region homologous to the R domain. In MDR, the interval between the first NBD and the second membrane-spanning domain is approximately 130 amino acids shorter than in CFTR. Sequence alignment suggested that a transition from one domain to another extended from about amino acid 708 to amino acid 835 in CFTR; this segment of 128 amino acids has no homology with MDR, but contains six of the eight consensus phosphorylation sites for cAMP-dependent protein kinase that occur in the sequence encoded by exon 13.
11. The R domain deletion was constructed in the vaccinia expression system plasmid pTM-CFTR4 (*20*) by the method of L. Kunkel [*Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985)]. The oligonucleotide hybridized to bases 2658 to 2638 and 2253 to 2233 of the CFTR cDNA, thereby deleting bases 2254 through 2637 of the CFTR cDNA. Amino acid residues of CFTR are numbered according to (*3*); numbering is for CFTR, not CFTRΔR.
12. We used the vaccinia virus–T7 hybrid expression system [O. Elroy-Stein, T. R. Fuerst, B. Moss, *ibid.* **86**, 6126 (1989)] to express CFTR or mutant forms in HeLa cells (*4, 6, 7*).
13. Cells were loaded with SPQ by including 10 mM SPQ in the media for 9 to 12 hours. SPQ fluorescence was initially quenched by incubating cells for 25 to 45 min in a buffer containing 135 mM NaI, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaSO_4$, 10 mM Hepes (pH 7.4), and 10 mM dextrose. We used I^- to assay Cl^- channels because it yields a better signal to noise ratio than Cl^-. After measuring fluorescence for at least 2 min, the 135 mM NaI solution was replaced by one containing 135 mM $NaNO_3$ (at 0 min) and fluorescence was measured for another 17.5 min. Forskolin (20 μM) and IBMX (100 μM) were added 5 min after the anion substitution. Fluorescence of SPQ in single cells was measured as described (*4, 6*). In each experiment, 12 to 38 cells were studied; experiments were performed in triplicate on each transfected culture; and at least three different transfected cultures were studied for each condition. Data are representative of responses obtained in each condition. Because the expression system produces a heterogeneous response (that is, not all cells express CFTR) (*4, 6*), the data shown are for the 40% of cells in each field with the largest response. Data are presented as the fluorescence at time t (F_t) minus the baseline fluorescence (F_o), the average fluorescence measured in the presence of I^- for 2 min prior to ion substitution. The rate of change in fluorescence is the critical variable in assessing anion permeability; differences between groups in absolute values of fluorescence may reflect quantitative differences between groups in SPQ loading, size of cells, or number of cells studied [N. P. Illsley and A. S. Verkman, *Biochemistry* **26**, 1215 (1987)].
14. Similarly, the NH_2-terminus of the *Drosophila Shaker* K^+ channel controls channel inactivation, perhaps by acting as a plug [T. Hoshi, W. N. Zagotta, R. W. Aldrich, *Science* **250**, 533 (1990); W. N. Zagotta, T. Hoshi, R. W. Aldrich, *ibid.*, p. 568]. However, when we coexpressed the R domain and CFTRΔR, the R domain peptide did not reverse the effects of deleting the R domain in CFTRΔR.
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22. We did not make mutations in equivalent amino acids in NBD1 and NBD2 because we know of no such paired mutations that are both nonfunctional and make mature, fully glycosylated CFTR (*20, 21*).
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