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Effects of Cystic Fibrosis and Congenital Bilateral Absence of the Vas Deferens–Associated Mutations on Cystic Fibrosis Transmembrane Conductance Regulator–Mediated Regulation of Separate Channels

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The protein defective in cystic fibrosis (CF), the CF transmembrane-conductance regulator (CFTR), functions as an epithelial chloride channel and as a regulator of separate ion channels. Although the consequences that disease-causing mutations have on the chloride-channel function have been studied extensively, little is known about the effects that mutations have on the regulatory function. To address this issue, we transiently expressed CFTR-bearing mutations associated with CF or its milder phenotype, congenital bilateral absence of the vas deferens, and determined whether mutant CFTR could regulate outwardly rectifying chloride channels (ORCCs). CFTR bearing a CF-associated mutation in the first nucleotide-binding domain (NBD1), ΔF508, functioned as a chloride channel but did not regulate ORCCs. However, CFTR bearing disease-associated mutations in other domains retained both functions, regardless of the associated phenotype. Thus, a relationship between loss of CFTR regulatory function and disease severity is evident for NBD1, a region of CFTR that appears important for regulation of separate channels.

Introduction

The autosomal recessive disorder cystic fibrosis (CF [MIM 219700]) manifests as chronic obstructive pulmonary disease, exocrine pancreatic deficiency, high content of sodium and chloride in sweat, and male infertility. These features are believed to be caused by abnormal electrolyte transport across epithelia, which leads to altered mucous viscosity and recurrent episodes of obstruction, inflammation, and progressive destruction of affected organs (Welsh et al. 1995). Initial electrophysiological studies of CF epithelial cells described the abnormal regulation of outwardly rectifying chloride channels (ORCCs); consequently, this abnormal regulation was implicated as the molecular defect underlying CF (Frizzell et al. 1986; Welsh and Liedtke 1986; Li et al. 1988; Hwang et al. 1989). Positional cloning of the gene responsible for CF provided a major breakthrough in the understanding of the molecular basis for this disease: CF is caused by mutations in the CF transmembrane-conductance regulator (CFTR [MIM 602421]) (Kerem et al. 1989; Riordan et al. 1989).

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CFTR is an integral membrane protein composed of two repeated units, each with a transmembrane domain (TMD) and cytoplasmic nucleotide-binding fold (NBF), separated by a regulatory (R) domain (Riordan et al. 1989). This protein functions as a cAMP-activated Cl^- channel, which meets the expectation that a chloride-transport defect is the underlying cause of CF (Drumm et al. 1990; Anderson et al. 1991; Bear et al. 1992). However, the Cl^- conduction properties of CFTR are different from properties of the ORCCs previously characterized in CF cells (Ward et al. 1991; Egan et al. 1992). An association between CFTR and ORCCs has been demonstrated by heterologous expression of CFTR in an epithelial cell line devoid of endogenous CFTR function. ORCC activity has been recovered in the complemented cells, and its regulation has been found to be similar to that in wild-type epithelial cells (Egan et al. 1992). This result implies either that CFTR forms more than one type of channel or that the ORCCs are a separate entity regulated by CFTR.

Three lines of evidence indicate that ORCCs are molecularly distinct from CFTR. First, functional measurements of ORCC density in plasma membranes do not correlate with the level of CFTR mRNA expression in several different epithelia cell lines (Ward et al. 1991). Second, ORCCs are present in nasal epithelial cells from mice that do not express CFTR (CF mice) but cannot be activated by cAMP-dependent protein kinase A (PKA [Gabriel et al. 1993]). Finally, reconstitution of four

proteins coprecipitated from bovine tracheal epithelia forms channels in lipid bilayers with physiological characteristics similar to those of the ORCCs and of CFTR. Immunodepletion of CFTR by an anti-CFTR antibody causes a loss of CFTR channels, and, although the ORCCs remain, they cannot be activated by PKA (Jovov et al. 1995). Thus, CFTR is a positive regulator of ORCCs, and activation of ORCCs contributes to the whole-cell chloride conductance in airway epithelial cells (Schwiebert et al. 1994).

CFTR is also a regulator of epithelial sodium channels. In patients with CF, increased absorption of sodium by respiratory epithelia compounds the difficulty in the hydrating of mucus secretions (Cotton et al. 1987). This electrophysiological abnormality has been attributed to CFTR dysfunction: the nasal epithelial cells from CF mice demonstrate Na^+ hyperabsorption, and heterologous expression of CFTR in primary CF airway cells normalizes Na^+ absorption (Grubb et al. 1994; Johnson et al. 1995). Functional cloning of the three subunits that form the amiloride-sensitive epithelial sodium channel (ENaC) enabled coexpression studies confirming the interaction between CFTR and this separate channel (Stutts et al. 1995). In respiratory epithelia, activated CFTR exerts a regulatory effect on ENaC, thereby reducing sodium absorption (Stutts et al. 1995; Mall et al. 1998). Likewise, sodium absorption via ENaC in sweat ducts requires functional CFTR (Reddy et al. 1999). Therefore, in addition to the conventional role of CFTR as a cAMP-activated chloride channel, the examples of ORCC and ENaC clearly illustrate that it is also a regulator of separate channels.

The effect that disease-associated CFTR mutations have on the activity of ORCCs has been studied as a proxy for the regulatory function of CFTR. Specifically, two missense mutations in NBF1 have been evaluated: A455E, which is associated with mild lung disease, and G551D, which is associated with a more severe pulmonary phenotype (Hamosh et al. 1992; Gan et al. 1995). CFTR bearing A455E retains both CFTR Cl^- channel activity and the ability to regulate ORCCs (Fulmer et al. 1995). In contrast, G551D-CFTR has some Cl^- channel activity but does not regulate ORCCs (Fulmer et al. 1995). It has been demonstrated that G551D-CFTR does not regulate the activity of ENaC (Ismailov et al. 1996). These results suggest that loss of the regulatory activity may coincide with the severity of lung disease. This hypothesis could explain the phenotypic variation between CF and congenital bilateral absence of the vas deferens (CBAVD [MIM 277180]). Even though CBAVD is caused primarily by mutations in CFTR, it is classified as a clinically distinct autosomal recessive disorder of male infertility in which lung disease is uncommon. Thus, we have studied the biosynthesis and function of CF- and CBAVD-associated mutations. Although the common CF mutation ΔF508 causes a loss of CFTR regulatory function, mutations in other regions permit CFTR regulation of ORCCs independent of the associated phenotype.

Methods

Mutation Analysis of Patients with CBAVD

Genomic DNA was isolated by standard phenol and chloroform extraction of proteinase K–digested leukocytes. DNA was assayed for 16 common CFTR mutations (R117H, 621+1G→T, R334W, R349P, A455E, ΔI507, ΔF508, 1717-1G→A, G542X, S549N, G551D, R553X, R560T, 3849+10 Kb C→T, W1282X, and N1303K), by reverse dot-blot hybridization (Mickle et al. 1998). In addition, 25 exons were screened by denaturing gradient gel electrophoresis, for mobility shifts, and, if necessary, dideoxy DNA sequencing was performed (Mickle et al. 1998). Exons 9 and 23 were sequenced directly.

Expression Analysis

The mutations ΔF508, R1070W, D1270N, and G1349D were created in the vector pBQ4.7 containing CFTR cDNA (pBQ4.7 is a gift from J. Rommens and L. C. Tsui), by single-stranded mutagenesis (Youssoufian et al. 1995), and then were shuttled into pRSV-CFTR, a Rous sarcoma virus (RSV)-driven expression plasmid, by use of *Kpn*2I and *Hpa*I (for ΔF508) or *Nco*I and *Sal*I (for R1070W, D1270N, and G1349D) restriction sites common to both plasmids (Fulmer et al. 1995). The mutations R1070P and R1070Q were created directly in pRSV-CFTR, by use of a transformer site–directed mutagenesis kit (Clontech). Immunoprecipitation experiments were performed, by use of the CFTR C-terminus–specific monoclonal antibody (Genzyme), on lysates of transiently transfected human embryonic kidney (HEK) 293 cells, detailed elsewhere (Mickle et al. 1998).

Patch-Clamp Analysis

Whole-cell patch-clamp recordings were performed on human airway (IB3-1) cells transiently transfected with either pRSV-CFTR or pRSV-CFTR/mutant, as described elsewhere (Mickle et al. 1998). IB3-1 bronchial epithelial cells were derived from a patient with CF (genotype ΔF508/W1282X); these cells lack functional CFTR (Zeitlin et al. 1991). To facilitate detection of transfected cells, IB3-1s were cotransfected with pTR-UF5 (Zolotukhin et al. 1996), a green-fluorescent protein (GFP) reporter plasmid, at a 10:1 ratio (pRSV-CFTR:pTR-UF5). CFTR function was not altered by GFP coexpression (Mickle et al. 1998). Symmetrical Tris-HCl solutions were used in the bath (145 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 5 mM *N*-2-hydroxyethylpiperazine-

N′-2-ethane-sulfonic acid [HEPES], 60 mM sucrose, and 1 mM $MgCl_2$) and in the pipette (145 mM Tris-Cl, pH 7.4, 5 mM HEPES, 5 mM Mg^{2+}-ATP, 100 nM $CaCl_2$, and 2.5 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetra-acetic acid) solutions (Schwiebert et al. 1994; Mickle et al. 1998). The holding potential was set at −60 mV. A voltage-clamp protocol was followed, stepping from −100 mV to +100 mV, at 20-mV increments, for a duration of 250 ms. Cells were pretreated with CPT-cAMP (8-[4-chlorophenylthio]; 200 μM) and forskolin (5 μM) for 5 min before the Cl^- channel blockers DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid; 500 μM) and glibenclamide (50 μM) were added to the bath solution. Data points from steady-state levels were taken to generate current-voltage (I-V) plots. Plots were fitted by Origin 4.0 (Microcal). Unpaired and paired Student's *t* tests were used to assess statistical significance of Cl^- currents between transfected and nontransfected cells and between positive and negative voltages, respectively; $P < .05$ was considered to be significant.

Results

Selection of CF- and CBAVD-Associated Mutations

To study the relationship between lung disease and CFTR function, we selected mutations that had been reported in at least two unrelated individuals with either CBAVD or CF. The clinical features and sweat tests are summarized, according to mutation, in table 1. Mutations at codon 1070 of TMD2 were selected, since two mutations (R1070P and R1070Q) have been associated with CF, whereas a third (R1070W) has been observed in men with CBAVD (table 1). The R1070W mutation was first reported by us to the Cystic Fibrosis Genetic Analysis Consortium. The index case was a 3-mo-old boy who, during surgery for a right inguinal hernia and hydrocele, was discovered to have bilateral absence of the vas deferens. Sweat Cl^- tests were normal at age 3 mo (14 mmol/liter and 16 mmol/liter) and at 9 mo (17 mmol/liter and 15 mmol/liter). At age 6 years, the patient was pancreatic sufficient and was growing normally, with no pulmonary problems. CFTR-mutation screening revealed that the proband was compound heterozygous for the common mutation ΔF508 and a C→T transition at nucleotide 3340. The latter mutation is predicted to change the amino acid at residue 1070 from arginine to tryptophan and is designated "R1070W." Also selected for study were two mutations in NBF2—D1270N and G1349D, which are associated with different pulmonary phenotypes (table 1). The mutation D1270N has been identified in at least nine men with CBAVD. For comparison purposes, we selected the CF-associated mutation G1349D, since it occurs in the same functional domain as does D1270N.

Biosynthesis of CFTR Missense Mutants

A number of CF mutations have been shown to affect the maturation of CFTR (Cheng et al. 1990). To determine whether the selected missense mutations affect CFTR biosynthesis, HEK 293 cells were transiently transfected with plasmid containing mutant CFTR cDNA, and then each mutant was immunoprecipitated by use of a monoclonal antibody specific for the carboxy terminus of CFTR. CFTR was detected by PKA-mediated ^{32}P radiolabeling followed by SDS-PAGE and autoradiography. Molecular mass was determined by comparison with commercially available molecular markers (RPN800; Amersham). Endogenous CFTR could not be detected in untransfected or mock (pTR-UF5)-transfected HEK 293 cells.

Wild-type CFTR migrated as a single band, at a molecular mass of ~175 kD (fig. 1; Cheng et al. 1990; Gregory et al. 1990). The 175-kD protein is the mature form of CFTR and is designated as "band C" in figure 1. The NBF1 mutant ΔF508-CFTR migrated as two bands, at ~145 kD (fig. 1, band B) and ~130 kD (fig. 1, band A). Band B is partially glycosylated protein; whether band A is unglycosylated protein or some other form is unclear (Cheng et al. 1990; Gregory et al. 1991). Despite several attempts, we were unable to identify the fully glycosylated, mature form of CFTR (i.e., band C) for ΔF508-CFTR.

All three missense mutations at arginine 1070 affected CFTR processing (fig. 1). R1070P(CF), like ΔF508, migrated as bands B and A, but the fully glycosylated form of CFTR (band C) was not observed. The R1070Q(CF) and R1070W(CBAVD) mutants consistently migrated as two bands; a minor fraction was partially glycosylated (band B), whereas the majority was mature CFTR (band C). Thus, mutations R1070Q and R1070W altered but did not prohibit complex glycosylation. The NBF2 mutants D1270N(CBAVD) and G1349D(CF) migrated like wild-type CFTR (band C), leading to the conclusion that neither mutation affects CFTR glycosylation (fig. 1).

Cl^- Channel and Regulatory Functions of CFTR Missense Mutants

To assay each CFTR function, plasmids containing wild-type CFTR, the ΔF508 mutant, and the missense mutants in TMD2 and NBF2 were transiently expressed in CF airway epithelial cells (IB3-1). This human cell line was chosen because IB3-1 cells express ORCCs but no functional CFTR (Egan et al. 1992). Whole-cell patch-clamp recordings were performed under conditions in which Cl^- was the predominant permeating anion in pipette (intracellular) and bath (extracellular) so-

Table 1

CFTR Missense Mutations and Associated Phenotypes

Mutation (Total No. of Cases)	No. of Cases										
	Lung Status			Pancreatic Status			Sweat Cl^-		Fertility		
	Normal[a]	Abnormal	Not Reported	Sufficient	Insufficient	Not Reported	Reported (Mean ± SEM [mmol/liter[b]])	Not Reported	Subfertile[c]	Not Reported	Phenotype
R1070W (7)[d]	5	0	2	5	0	2[e]	6 (50.2 ± 13.4)	1	6	1	CBAVD
R1070P (2)[f]	0	1	1	0	1	1	1 (Positive)	1	0	2	CF
R1070Q (14)[g]	0	7	7	0	7	7	7 (Positive)	7	2	12	CF
D1270N (9)[h]	4	0	5	4	0	5	3 (77.5 ± 16.7)	6	3	6	CBAVD
G1349D (3)[i]	0	0	3	0	0	3	…	3	1	2	CF

[a] No history of chronic lung disease.

[b] Concentrations >60 mmol/liter are diagnostic of CF (i.e., positive).

[c] Decreased reproductive fitness. Although CBAVD occurs in men, the potential for assisted reproductive interventions precludes a designation of "infertile."

[d] Data are from Casals et al. (1995), Chillón et al. (1995), Ferec et al. (1995), Jezequel et al. (1995), Le Lannou et al. (1995), Gervais et al. (1996), de la Taille et al. (1998), and the present study.

[e] In one case, a newborn tested positive for immunoreactive trypsinogen.

[f] Data are from Shrimpton et al. (1997) and T. Doerk (personal communication).

[g] Data are from Audrézet et al. (1993), Mercier et al. (1993, 1994), Osborne et al. (1993), Savov et al. (1994), Bienvenu et al. (1997), and Estivill et al. (1997).

[h] Data are from Anguiano et al. (1992), Claustres et al. (1993), Verlingue et al. (1993), Casals et al. (1995), Chillón et al. (1995), Mercier et al. (1995), Estivill et al. (1997), Bombieri et al. (1998), and Fanen et al. (1999).

[i] Data are from Beaudet et al. (1991), Gregory et al. (1991), Anderson and Welsh (1992), Welsh and Smith (1993), Chillón et al. (1995), and Estivill et al. (1997). The initial mutation report (Beaudet et al. 1991) indentified G1349D on two CF chromosomes, and, on the basis of these cases, G1349D has been described as a CF-associated mutation (Gregory et al. 1991; Anderson and Welsh 1992), with specific reference to PI (Welsh and Smith 1993).

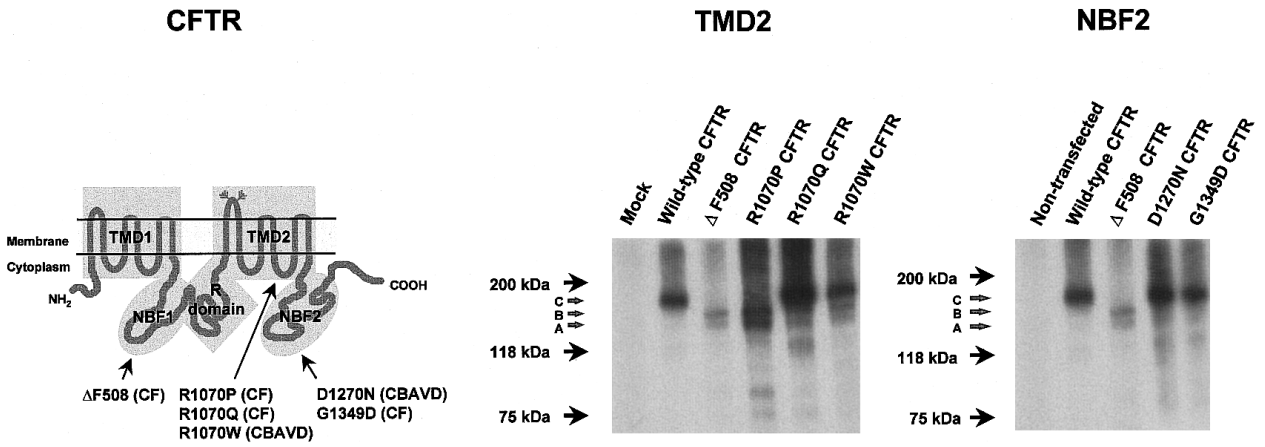

Figure 1 Immunoprecipitation of TMD2 and NBF2 mutants transiently expressed in HEK 293 cells. The left panel is a cartoon of CFTR and the relative location of mutations studied in this report; domains of CFTR are described in the text. The middle and right panels are autoradiographs of wild-type and mutant CFTR proteins that were expressed in HEK 293 cells, immunoprecipitated with a monoclonal antibody directed against the C-terminus, radiolabeled with ^{32}P by use of PKA, and electrophoretically separated by SDS-PAGE. Wild-type CFTR was fully processed (bands C). The ΔF508-CFTR is a processing mutant that migrated as two bands (bands B and bands A). The TMD2 mutants affected processing to differing degrees: R1070Q(CF) and R1070W(CBAVD) migrated as bands C and B, whereas R1070P(CF) migrated as bands B and A. The NBF2 mutants D1270N(CBAVD) and G1349D(CF) migrated as band C. "Mock" denotes cells transfected with the GFP plasmid pTR-UF5. Apparent mass of the proteins, as described in the text, was determined with the RPN800 (Amersham) size markers run in parallel.

lutions. Currents from cells expressing wild-type or mutant CFTR were significantly greater in magnitude than were the currents recorded from either nontransfected cells or cells expressing only GFP ($P < .05$, unpaired Student's t test; fig. 2). Each of the missense mutants generated robust Cl^- currents comparable to those of wild-type CFTR (fig. 2). Moreover, Cl^- currents from cells expressing wild-type CFTR and missense mutants were significantly greater at positive than at negative voltages ($P < .05$, paired Student's t test), indicating that their currents were outwardly rectified (fig. 2). Therefore, each TMD2 and NBF2 mutant that we studied generated currents that were similar, in magnitude and rectification, to those of wild-type CFTR. In contrast, cells transfected with ΔF508-CFTR produced significantly lower Cl^- currents than did those of cells expressing either wild-type CFTR or any one of the missense mutants ($P < .05$). Furthermore, the current was not significantly rectified for ΔF508-CFTR ($P > .05$ for ICl^- at +100 mV vs. −100 mV; fig. 2).

Specific Cl^- channel inhibitors were used to establish whether the observed whole-cell Cl^- currents were due to activation of CFTR channels, other ion channels, or both. Stimulated cells expressing wild-type CFTR display an outwardly rectifying profile (fig. 3*A*, *blackened circles*). The stilbene derivative DIDS inhibits Cl^- channels such as ORCCs and anion exchangers but does

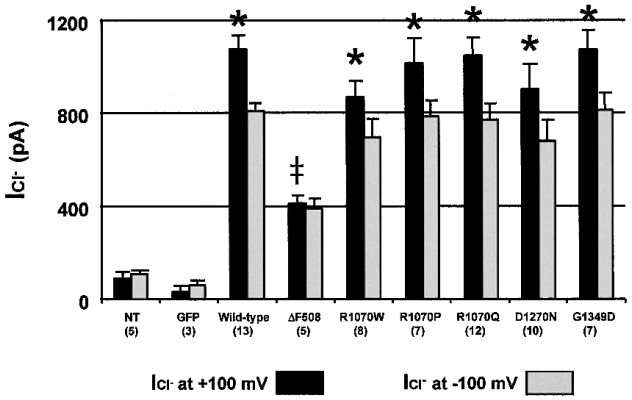

Figure 2 cAMP-stimulated whole-cell chloride currents (ICl^-) recorded from IB3-1 airway cells overexpressing wild-type and mutant CFTR. The graph presents mean Cl^- currents and standard errors of the mean (*error bars*) at +100 mV (*black bars*) and -100 mV (*gray bars*). Currents at 250 ms are shown for all recordings. The number of cells patched is indicated in parentheses. For each classification, the difference between the current magnitude displayed at positive and negative voltages is attributed to ORCC function; an asterisk indicates Cl^- currents at positive voltages (+100 mV) that were significantly greater than the Cl^- currents at negative voltages (−100 mV) ($P < .05$). A double cross indicates current amplitudes of mutant CFTR that were significantly lower than those of wild-type CFTR ($P < .05$). NT = not transfected.

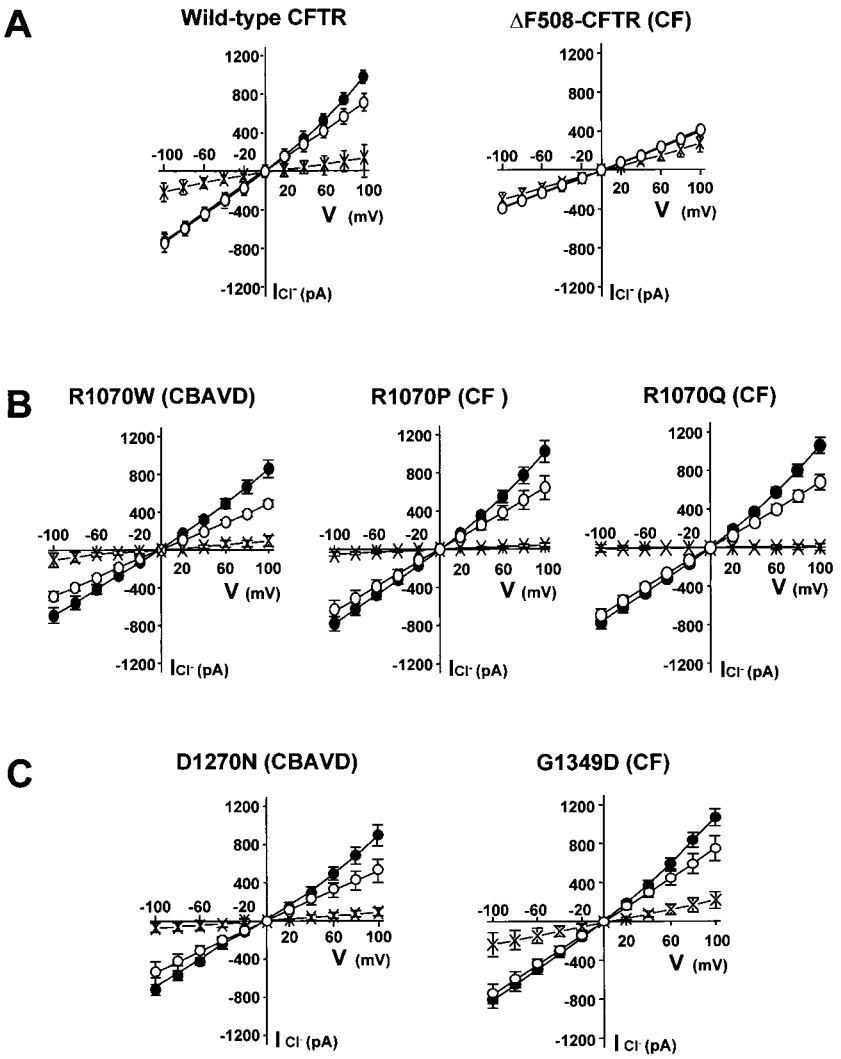

Figure 3 Current-voltage plots of whole-cell patch-clamp data from airway cells transfected with wild-type CFTR and disease-associated mutants. Mean whole-cell Cl^- currents at 250 ms, as well as standard errors of the mean (*error bars*), were calculated for each voltage step. These means were plotted against the voltage steps, to produce the I-V plots. *A,* cAMP-stimulated IB3-1 cells overexpressing wild-type CFTR, which generated outwardly rectifying Cl^- current (*blackened circles*). DIDS (500 μM) eliminated the rectification, leaving a linear I-V profile (*unblackened circles*). Glibenclamide (50 μM) blocked CFTR; the remaining current was similar to that in nontransfected cells (*crosses*). The NBF1 mutant ΔF508 generated a linear conductance that was insensitive to DIDS (*overlapping blackened and unblackened circles*) but responded to glibenclamide (*crosses*). *B,* CBAVD(R1070W)- and CF(R1070P and R1070Q)–associated mutants in TMD2 had I-V plots similar to those of wild-type CFTR: outwardly rectified currents (*blackened circles*) that responded to DIDS (*unblackened circles*) and glibenclamide (*crosses*). *C,* CBAVD(D1270N) and CF(G1349D) mutations in NBF2 had I-V profiles comparable to those of wild-type CFTR.

not inhibit CFTR Cl^- channel activity (Tilmann et al. 1991; Egan et al. 1992; Gabriel et al. 1993; Schwiebert et al. 1994; Mastrocola et al. 1998; Lee et al. 1999). The extracellular addition of DIDS to cells expressing wild-type CFTR eliminated the outwardly rectified component of the Cl^- currents. The remaining current had a linear I-V relationship (fig. 3*A, unblackened circles*). Glibenclamide inhibits CFTR Cl^- currents (Sheppard and Welsh 1993; Schwiebert et al. 1994, 1995; Fulmer et al. 1995). Application of glibenclamide significantly reduced the linear DIDS-insensitive Cl^- currents, indicating that the current was due to CFTR Cl^- channel activity (fig. 3*A, crosses*). The residual DIDS- and glibenclamide-insensitive conductance (+100 mV, 140.5 ± 130.0 pA; -100 mV, −214.8 ± 97.5 pA; n = 3) is similar to that observed for nontransfected cells and likely represents current from other endogenous Cl^- channels. Thus, the combination of I-V relationship and response to inhibitors allowed dissection of whole-cell Cl^- currents into two components: outwardly rectified and DIDS sensitive, carried by separate channels such as ORCCs; and linear, DIDS insensitive, and gli-

Table 2

Summary of Processing and Whole-Cell Function of CFTR Mutants

Domain and Mutation	Phenotype	CFTR Status[a]				
		Processing[b]			Function	
		Band A	Band B	Band C	Cl^- Channel	Regulatory[c]
Not applicable:						
Wild type	Normal	−	−	+++	+++	+
NBF1:[d]						
A455E[e]	CF[e]	+	++	−	+++	+
ΔF508	CF	+	++	−	+	−
G551D	CF	−	−	+++	+	−
TMD2:						
R1070W	CBAVD	−	+	++	+++	+
R1070P	CF	+	++	−	+++	+
R1070Q	CF	−	+	++	+++	+
NBF2:						
D1270N	CBAVD	−	−	+++	+++	+
G1349D	CF	−	−	+++	+++	+

[a] A minus sign (−) denotes absence; a single plus sign (+) denotes "low"; a double plus sign (++) denotes "intermediate"; and a triple plus sign denotes "high."

[b] Band designations are as in figure 1.

[c] Data are for regulation of DIDS-sensitive chloride channels (e.g., ORCCs).

[d] Data for A455E and G551D have been reported elsewhere (Fulmer et al. 1995).

[e] Associated with mild lung disease (Gan et al. 1995).

benclamide responsive, generated by CFTR Cl^- channels.

In contrast to those of wild-type CFTR, the currents generated from cells expressing the CF-associated mutant ΔF508 were neither outwardly rectifying nor DIDS sensitive (fig. 3*A*, overlap of blackened and unblackened circles) but were reduced by glibenclamide (fig. 3*A*, *crosses*). Thus, ΔF508-CFTR functioned as a Cl^- channel but lacked regulatory function. For each missense mutant that we studied, the application of DIDS eliminated 30%–40% of the whole-cell Cl^- current at +100 mV ($P < .05$, paired Student's t test). Both the CBAVD mutant (R1070W) and the CF mutants (R1070P and R1070Q) in TMD2 generated outwardly rectified Cl^- currents that were inhibited by DIDS (fig. 3*B*). In fact, the I-V and inhibition profiles of all three R1070 mutants were similar to those of wild-type CFTR (fig. 3*B*). Likewise, cells expressing either of the NBF2 mutants—D1270N (CBAVD) and G1349D (CF)—had both components of whole-cell Cl^- currents (fig. 3*C*). Glibenclamide reduced currents of each mutant to the same degree as was observed in cells transfected with wild-type CFTR. Thus, transient expression of the TMD2 and the NBF2 mutants restored both CFTR Cl^- channel and regulatory activities, regardless of the associated phenotypes.

Discussion

In this article, we have addressed the question of whether loss of CFTR regulatory function coincides with the pulmonary phenotype. To test this hypothesis, we evaluated the effect that CF- and CBAVD-associated mutations have on both CFTR processing and whole-cell function (table 2). Our results indicate that processing abnormalities do not correlate directly with functional defects. We were unable to detect mature glycosylated forms for two mutants, ΔF508 and R1070P; yet, each of these generated Cl^- currents attributed to CFTR activity. The latter observation indicates that a certain amount of functional CFTR was present in the plasma membrane, despite the absence of detectable mature protein. One possibility that may account for this observation is that CFTR is handled differently in the cells used for functional analyses (IB3-1 cells) than in the cells used to evaluate processing (HEK 293 cells). Although both cell lines are models for human epithelial-cell studies, CFTR in IB3-1 cells is not expressed at levels high enough to be reliably detected by immunoprecipitation. Thus, CFTR processing was evaluated in HEK 293 cells. Our results are comparable to those published by others who used HEK 293 cells (Seibert et al. 1996). In addition, other investigators have consistently observed that transiently overexpressed ΔF508-CFTR retains partial Cl^- channel activity in a variety of cell types (Dalemans et al. 1991; Drumm et al. 1991; Denning et al. 1992; Cheng et al. 1995; Frizzell 1995). Thus, incompletely glycosylated "immature" forms of CFTR may be functional, and, when overexpressed, they may reach the cell membrane. Alternatively, fully processed forms of CFTR may have been present but below the limit of detection by immunoprecipitation.

To probe the interaction between CFTR and separate channels, it was necessary to study CFTR in an intact cell system. The airway cell line IB3-1, which is devoid of functional CFTR, was selected because complementation with the wild-type protein restores both CFTR Cl^- channel activity and CFTR-mediated regulation of ORCCs (Egan et al. 1992). However, transient overexpression of the heterologous protein may have concealed the effect that a mutation has on either processing or function. One could argue that this situation occurred in our analysis of the CFTR regulatory function: certain mutations affected CFTR regulation, but, when overexpressed, they appeared to have normal regulatory function. Although this possibility cannot be excluded, CFTR regulation of ORCCs is evident over a very wide range of CFTR expression levels (Ward et al. 1991). Moreover, an intermediate level of CFTR regulatory function was not observed for any mutant; regulation of ORCCs was either present or absent. Thus, the CFTR regulatory function may be an all-or-none phenomenon that is independent of CFTR expression level.

Our study of disease-associated mutations suggests that CFTR regulatory function is mediated by a specific region. For example, CF-associated mutations in the NBF1 domain, ΔF508 (present study), and G551D (Fulmer et al. 1995), ablated CFTR regulation of ORCCs, whereas the regulatory ability was not impaired by mutations in TMD2 and NBF2 (table 2). Of particular note are the results obtained with the NBF2 mutation G1349D. The latter mutation is comparable, in terms of nature (glycine to aspartic acid) and location (Walker C motif), to the NBF1 mutation G551D; yet, CFTR bearing G1349D-generated DIDS-sensitive currents that were attributed to the regulation of ORCCs. These findings are consistent with functional analyses of synthetic CFTR truncations that indicate that the region encompassing NBF1 and the R domain is necessary for CFTR to regulate ORCCs (Schwiebert et al. 1998). Furthermore, the same region appears to mediate CFTR regulation of ENaC (Schreiberet al. 1999). Yeast two-hybrid analysis suggests a direct interaction between NBF1 and the α subunit of ENaC, and also ΔF508-CFTR is unable to regulate ENaC (Mall et al. 1996; Kunzelmann et al. 1997). Likewise, NBF1 appears to be necessary for the CFTR-ROMK2 interaction that confers sulfonylurea sensitivity (McNicholas et al. 1997). Together, these observations indicate that NBF1 is critical for CFTR regulation of separate channels.

The importance of the regulatory function in the development of the CF phenotype is unknown. Although synthetic constructs demonstrate that CFTR Cl^- channel and ORCC regulation were separately mutable, disease-associated mutations that abolish the regulatory function without altering the Cl^- channel activity have not been reported. This may be due to ascertainment bias: mutations that affect only the regulatory function might produce a phenotype different from either CF or CBAVD. Alternatively, CFTR regulation of separate channels may contribute to the coordination of ion movement across epithelia in the healthy state, but this function may become inconsequential when CFTR Cl^- channel activity is altered. Finally, phenotypic implications of the regulatory function may occur for only a subset of CFTR mutations, specifically those which affect the region responsible for regulation. This could explain the correlation of pulmonary phenotype with ORCC regulation for mutations in NBF1 and the lack of correlation for mutations in other regions.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Cystic Fibrosis Genetic Analysis Consortium, http://www.genet.sickkids.on.ca/CFTR (for R1070W)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nim.nih.gov/Omim (for CF [MIM 219700], CFTR [MIM 602421], and CBAVD [MIM 277180])

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