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Maturation and Function of Cystic Fibrosis Transmembrane Conductance Regulator Variants Bearing Mutations in Putative Nucleotide-Binding Domains 1 and 2

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One feature of the mutations thus far found to be associated with the disease cystic fibrosis (CF) is that many of them are clustered within the first nucleotide-binding domain (NBD) of the CF transmembrane conductance regulator (CFTR). We sought to discover the molecular basis for this clustering by introducing into the two NBDs of CFTR mutations either mimicking amino acid changes associated with CF or altering residues within highly conserved motifs. Synthesis and maturation of the mutant CFTR were studied by transient expression in COS cells. The ability of the altered proteins to generate cyclic AMP-stimulated anion efflux was assessed by using 6-methoxy-*N*-(sulfopropyl) quinolinium (SPQ) fluorescence measurements in HeLa cells expressing mutated plasmids. The results show that (i) all CF-associated mutants, with one exception, lack functional activity as measured in the SPQ assay, (ii) mutations in NBD1 are more sensitive to the effects of the same amino acid change than are the corresponding mutations in NBD2, (iii) cells transfected with plasmids bearing CF-associated mutations commonly but not exclusively lack mature CFTR, (iv) NBD mutants lacking mature CFTR fail to activate Cl^- channels, and (v) the glycosylation of CFTR, per se, is not required for CFTR function. We reason that the structure of NBD1 itself or of the surrounding domains renders it particularly sensitive to mutational changes. As a result, most NBD1 mutants, but only a few NBD2 mutants, fail to mature or lack functional activity. These findings are consistent with the observed uneven distribution of CFTR missense mutations between NBD1 and NBD2 of CF patients.

Mutations in a gene encoding a protein called the cystic fibrosis transmembrane conductance regulator (CFTR) are responsible for the disease cystic fibrosis (CF) (14, 25, 26). This was confirmed by showing that transfected full-length cDNA encoding CFTR was able to complement the defect in Cl^- channel regulation which is characteristic of epithelial cells from CF patients (8, 24). Although the protein product of the CF-associated gene has been identified, little is known about its biochemical function. Data obtained to date are consistent with the prediction that CFTR is a membrane-associated glycoprotein which can be phosphorylated in vitro (3, 9).

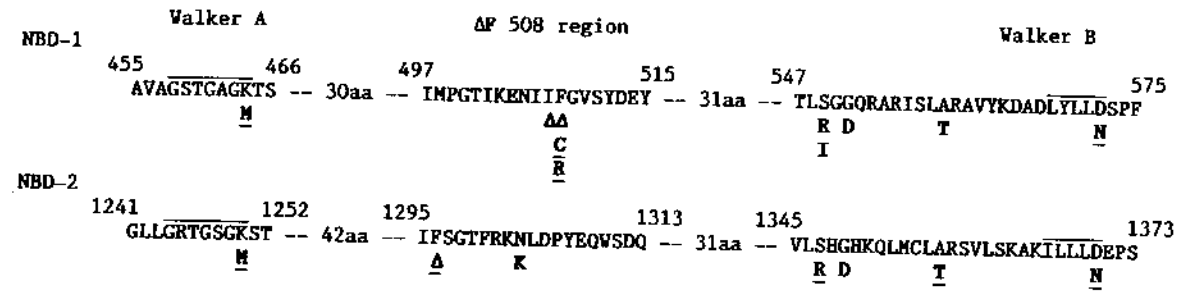
Amino acid sequence analysis and homology with other proteins suggest that CFTR consists of two repeats of a unit containing six membrane-spanning segments and a putative nucleotide-binding fold (25). The two repeats are separated by a large polar segment called the R (regulatory)-domain because it contains multiple potential phosphorylation sites. The overall structure of CFTR, with the exception of the R-domain, resembles that of a number of other integral membrane proteins such as the human and mouse multidrug resistance P-glycoproteins (MDR) and the protein encoded by the yeast *STE6* gene. Models for the structure of the nucleotide-binding folds have been proposed (12, 21).

Recent studies showed that the expression of CFTR in a variety of heterologous cells generates cyclic AMP (cAMP)-activated Cl^- currents which were not present in untransfected cells (1, 13). The simplest interpretation of these results is that CFTR is itself a cAMP-activated Cl^- channel. The definitive demonstration of this will require additional experiments, such as the use of isolated lipid bilayers containing highly purified CFTR or by mutagenesis of amino acids potentially involved in ion selectivity.

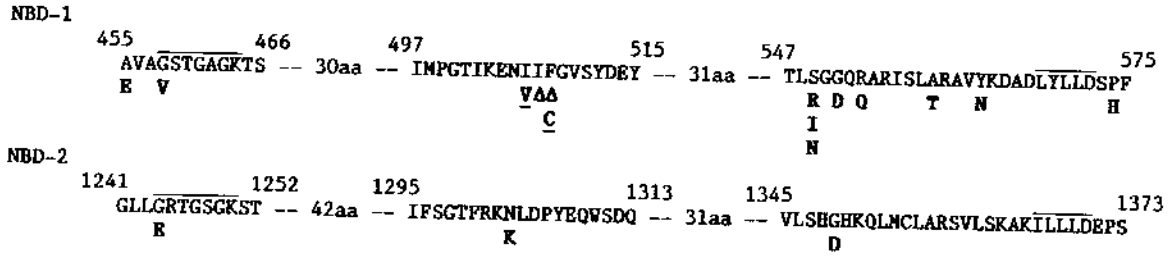
Studies of the biosynthesis of CFTR in transfected cells identified a defect associated with a number of CF mutations, including the most common, ΔF508 (3). Following synthesis, wild-type CFTR was shown to be processed by addition of complex carbohydrate. By contrast, several mutated versions of CFTR failed to yield the fully glycosylated form of the protein (referred to as band C). It was proposed that addition of carbohydrate to wild-type CFTR occurs in a two-step process: partial glycosylation in the endoplasmic reticulum, followed by complete glycosylation in the Golgi. Further, because mutations cause structural alterations, some forms of mutant CFTR are recognized as abnormal and rejected by a quality control mechanism located in the endoplasmic reticulum. Consequently, they are unable to move through the normal intracellular trafficking pathway. As a result, such mutants do not reach the Golgi, are not fully glycosylated, and are observed as an immature, partially glycosylated form called band B. This process of quality control is common to a number of secreted and transmembrane proteins, which when incorrectly folded or assembled fail to progress through the secretory pathway (11, 16, 20, 23, 27). In the case of CFTR, this interpretation

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A.



B.



C.



FIG. 1. Amino acid sequence alignments of NBD1 and NBD2. (A) Amino acid sequences, shown in single-letter code. The mutants studied around Walker A and B and F508 are given below the wild-type sequence. Underlined amino acids are not known to be associated with CF. Bars above the sequences indicate the highly conserved motifs. Δ, deletion of an amino acid. Numbers above the amino acids refer to the sequence published by Riordan et al. (25). (B) Amino acid changes reported to be associated with CF (4–7, 15, 16, 22, 25, 26). The underlined residues are reported natural polymorphisms. Mutations thought to be splice mutations rather than missense mutations are not shown. (C) Alignment of putative NBD amino acid sequences from MDR species, STE6, HYLB, and CFTR showing an alternative alignment of NBD2 of CFTR which maximizes sequence homology with other sequences shown. *, amino acid residues that are highly conserved in this family of proteins. H, human; M, mouse; PF, *Plasmodium falciparum*; HYLB, *Escherichia coli* hemolysin B. A consequence of this alignment is that F508 does not exactly correspond with F1296.

would mean that most cases of CF are caused by the absence of mature CFTR at the correct cellular location. Although no accurate localization of endogenous CFTR has been reported, immunolocalization of wild-type and mutant CFTR in recombinant cells is consistent with this hypothesis (3), and functional assays strongly suggest that at least some CFTR is present in the plasma membrane at the apical surface (32).

One interesting observation arising from studies on CF-associated mutations is the preponderance of identified mutations which map to nucleotide-binding domain 1 (NBD1) as opposed to NBD2 (4–7, 15, 17, 22) (Fig. 1). In addition, we recently showed that mutation of a homologous amino acid residue within NBDs 1 and 2 of CFTR resulted in different effects on CFTR maturation (3). These observations suggested to us that the frequency of CF-associated mutations within the NBDs might reflect an underlying sensitivity of NBD1 to functional inactivation or maturational blockage. To examine this possibility, we have studied not only the maturation of additional mutant CFTR molecules but also their function as measured by halide ion efflux in transfected cells. The studies were designed to determine how mutations affect function and how equivalent mutations in the two NBDs affect both processing and Cl^- transport. We hoped, thereby, to learn the contributions that the two NBDs make to CFTR function and to determine the molecular basis of the uneven distribution of mutations associated with CF between the amino-terminal and carboxy-terminal NBDs. To this end, we have introduced paired mutations into a number of sites within the two NBDs of CFTR. Some

mutations were introduced into residues altered in the DNA of CF patients, whereas others were in amino acids conserved in other NBD-containing proteins.

MATERIALS AND METHODS

In vitro mutagenesis procedures and plasmid constructions. Site-directed mutagenesis of CFTR and construction of expression vectors were performed as described previously (3), using standard methods for recombinant DNA manipulations and oligonucleotide-directed mutagenesis (18, 28). Briefly, pMT-CFTR, a plasmid vector for CFTR expression in mammalian cells, was constructed by placing CFTR cDNA sequences from the *Ava*I site at position 122 in the cDNA sequence to the *Sac*I site at position 4620 into the unique *Bgl*II site of the expression vector pSC-CEV1, using synthetic adaptor sequences (3). In pMT-CFTR, expression of CFTR is controlled by the flanking mouse metallothionein I promoter and simian virus 40 early polyadenylation signal. The previously described high-copy-number CFTR plasmid pTM-CFTR-4 was used for all site-directed mutagenesis procedures (3). CFTR mutations were verified by DNA sequencing, restriction analysis, and in vitro translation. Mutants constructed in the pTM-CFTR-4 background were directly tested for activity in the 6-methoxy-*N*-(sulfopropyl) quinolinium (SPQ) assay as described below. For expression in COS-7 cells, CFTR cDNA mutants were transferred to pMT-CFTR as described previously (3). Mutations occurring before codon 1270 of CFTR were transferred from pTM-CFTR-4 to pMT-CFTR on a 3.5-kb *Xba*I-to-*Bst*XI restriction fragment, and those occurring after codon 1270 were transferred on a 0.65-kb *Bst*XI-to-*Xho*I restriction fragment. Transient expression of CFTR in COS-7 cells was performed essentially as described elsewhere (28).

Immunoprecipitations and protein phosphorylation using protein kinase A. Our procedures for preparation of cell lysates, immunoprecipitation of CFTR, in vitro phosphorylation, and SDS (sodium dodecyl sulfate)-gel electrophoresis have all been described elsewhere (3, 9). For in vitro labeling of CFTR, immunoprecipitates were incubated with 20 ng of protein kinase A (Sigma) and 10 μCi of [γ-^{32}P]ATP in 50 μl of kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 100 μg of bovine serum albumin per ml) at 30°C for 60 min. The reaction was stopped by the addition of 0.5 ml of RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Labelled CFTR immunoprecipitates were analyzed by electrophoresis on 6% SDS-polyacrylamide gels (19).

Assessment of anion permeability. Our procedures for expression of CFTR by using the vaccinia-T7 hybrid expression system and for assessment of Cl^- permeability by using the halide-sensitive fluorophore SPQ have been described in detail elsewhere (1, 24). Briefly, HeLa cells were infected with recombinant vaccinia virus vTF7-3 (multiplicity of infection of 10 to 20) to produce bacteriophage T7 RNA polymerase. Following infection, the cells were transfected with plasmid (5 μg) containing wild-type or mutant CFTR coding sequences preceded by the T7 promoter. Cells were loaded with SPQ by including the dye in the medium and were studied 8 to 14 h after transfection. SPQ fluorescence was quenched by equilibration of the cells in NaI buffer. Cells were stimulated by addition of 20 μM forskolin and 100 μM isobutylmethyl xanthine to increase intracellular cAMP. SPQ fluorescence was measured before and following substitution of I^- for NO_3^- in the bathing medium. The change in SPQ fluorescence of wild-type CFTR under cAMP-stimulated and unstimulated conditions was included as a control for each mutant. Data are presented as percent fluorescence, which is $100 \times [(F_t - F_0)/(F_{17.5} - F_0)]$, where F_0, $F_{17.5}$, and F_t are fluorescence intensities at time zero, 17.5 min, and time t, respectively.

In a few studies, we also measured membrane currents with the standard whole-cell patch clamp technique (1, 10). For G1349D CFTR mutants, the pipette contained 120 mM *N*-methyl-D-glucamine (NMDG), 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 3 mM $MgCl_2$, 1 mM CsEGTA, and 1 mM MgATP, pH 7.2 (117.5 mM HCl). The external bath solution contained 140 mM NMDG, 10 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM dextrose, pH 7.2 (135 mM HCl). Membrane voltage was maintained at −60 mV and depolarized to +80 mV for 500 ms every 4 s. For K1250M CFTR mutants, the pipette contained 120 mM NMDG, 115 mM aspartic acid, 5 mM HEPES, 3 mM $MgCl_2$, 1 mM CsEGTA, and 1 mM MgATP, pH 7.2 (5.8 mM HCl). Membrane voltage was maintained at −80 mV and depolarized to +80 mV for 500 ms every 4 s. HeLa cells were studied 12 to 15 h after transfection. Series resistance and cell capacitance were compensated as described previously (1). Cells were studied at 30 to 35°C.

RESULTS

CFTR mutants. The mutations introduced into the NBDs of CFTR cDNA are shown in Fig. 1A, together with the CF-associated mutations that have been identified in these regions of the protein (Fig. 1B). The NBD mutations fall into three subregions: (i) those around the first Walker motif (Walker A) (31), characterized by several conserved glycines and a lysine residue; (ii) those upstream of and including the second Walker motif (Walker B), which consists of a short stretch of hydrophobic residues followed by a conserved aspartic acid; and (iii) the region around the most common CF-associated mutation (ΔF508). The sequences around residue 508 in Fig. 1A and B are arranged differently from the alignment proposed by Riordan et al. (25). The alignment used here was generated to maximize homology of NBD2, not only with NBD1 of CFTR but also with the NBD regions of other related proteins such as MDR and STE6 (Fig. 1C). The new alignment reveals a greater degree of conservation than does that published earlier.

In the Walker A sequences, we mutated to methionine the conserved lysine residue at amino acid 464 in NBD1 and at 1250 in NBD2; neither of these mutations is known to be associated with CF. By convention, these mutations are referred to as K464M and K1250M, where K and M stand for lysine and methionine, respectively, using the single-letter amino acid code. Upstream of Walker B, we made the mutations S549R and S549I, both of which are CF associated but relatively rare (5, 15). In NBD2 we made the equivalent S1347R mutation, which is not known to be CF associated. Mutations G551D and G1349D map to the equivalent conserved glycine in NBD1 and -2, respectively, and both are reported to be CF associated (5, 16a). A559T is CF associated (5), but its NBD2 equivalent A1357T is not reported to be CF associated. D572N and D1370N are not CF associated but alter the conserved aspartic acid in the Walker B sequence.

In the 508 region we deleted phenylalanine residue 508 (ΔF508) and also made Δ1507, a rare CF-associated mutation (15), F508C, which is reported to be a normal human polymorphism (17), and F508R, which represents a nonconservative amino acid change that is not known to be CF

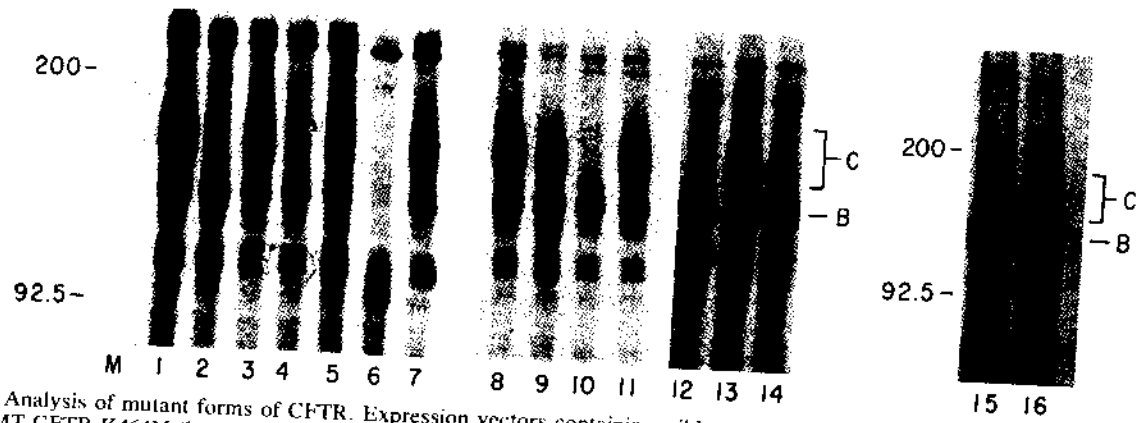


FIG. 2. Analysis of mutant forms of CFTR. Expression vectors containing wild-type CFTR (pMT-CFTR; lane 1) and those containing mutants pMT-CFTR-K464M (lane 2), pMT-CFTR-K1250M (lane 3), pMT-CFTR-S549R (lane 4), pMT-CFTR-S1347R (lane 5), pMT-CFTR-F508C (lane 7), pMT-CFTR-G551D (lane 8), pMT-CFTR-G1349D (lane 9), pMT-CFTR-A559T (lane 10), pMT-CFTR-A1357T (lane 11), pMT-CFTR-D572N (lane 12), pMT-CFTR-D1370N (lane 13), pMT-CFTR-N1303K (lane 14), pMT-CFTR-ΔF508 (lane 15), and pMT-CFTR-ΔF1296 (lane 16) were transfected into COS-7 cells, and CFTR expression was analyzed by immunoprecipitation, in vitro phosphorylation, and gel electrophoresis as described previously (3, 9). Lane 6 contains mock-transfected COS-7 cells.

associated (3). In NBD2 we made ΔF1296, which in the published amino acid alignment (25) is equivalent to the deletion at 508. In the same region of NBD2 we also made N1303K, which is CF associated (22). We also removed both potential glycosylation sites in CFTR to generate the mutation N894,900Q (3).

The mutations were introduced into a full-length CFTR cDNA by standard methods (18). Previously described vectors were used for CFTR mutagenesis and for CFTR expression in the vaccinia virus and COS cell systems (3, 9, 24).

Expression of mutant CFTR in COS cells. Figure 2 shows analysis of CFTR in COS cells 48 h after DNA transfection. CFTR was detected by addition of protein kinase A to washed immunoprecipitates made from extracts of the transfected cells, using monoclonal antibody 13.1 as described previously (3). The affinity of this antibody for CFTR is not measurably affected by either glycosylation of CFTR or the state of phosphorylation of the R-domain (2a, 3). Mature fully glycosylated CFTR is detected in this assay as a diffuse band (band C) migrating with an apparent molecular weight of 150,000 under our gel electrophoresis conditions. The primary translation product of CFTR, lacking any carbohydrate (band A), migrates more rapidly (apparent molecular weight of approximately 130,000) than band C, and partially glycosylated CFTR (band B) migrates with an intermediate mobility (apparent molecular weight of approximately 135,000).

Previously we reported that band C is absent from extracts of cells transfected with N894,900Q, the mutant lacking glycosylation sites (3). Instead the primary translation product, band A, is detected. Of mutations within NBD1, we previously showed that extracts from cells transfected with K464M, ΔI507, ΔF508, F508R, and S549I lack band C but contain band B. We also showed that extracts from cells transfected with NBD1 mutant G551D contain band C (3; Fig. 2). Figure 2 shows that the additional NBD1 mutants S549R, A559T, and D572N directed synthesis of band B but not band C CFTR. F508C, the NBD1 polymorphism, clearly makes band C, though possibly at somewhat reduced amounts.

Within NBD2, we had previously shown that cells containing mutant K1250M make band C (3; Fig. 2). Figure 2 shows that NBD2 mutants S1347R, G1349D, A1357T, and D1370N also direct synthesis of apparently normal levels of band C CFTR. However, extracts of cells transfected with NBD2 mutant N1303K contain only band B CFTR. Although in Fig. 2 little band C is visible in cells transfected with the ΔF1296 mutant of CFTR, additional experiments consistently showed that cells transfected with this mutant make small but detectable amounts of band C.

The expression data (summarized in Table 1) show that mutations in NBD1 are more sensitive to the effects of the same amino acid change than are mutations in NBD2, as

TABLE 1. Properties of CFTR mutants[a]

Mutant[b]	CF	Domain	Band			SPQ assay
			A	B	C	
Wild type			-	+	+	+[c]
K464M	N	NBD1	–	+	-	–
K1250M	N	NBD2	–	+	+	+[c]
ΔI507	Y	NBD1	–	+	-	–
ΔF508	Y	NBD1	–	+	–	–[c]
F508C	P	NBD1	-	+	+	+
F508R	N	NBD1	–	+	–	–
ΔF1296	N	NBD2	-	+	+	+
N1303K	Y	NBD2	–	+	–	–
S549I	Y	NBD1	-	+	-	-
S549R	Y	NBD1	–	+	–	–
S1347R	N	NBD2	-	+	+	+
G551D	Y	NBD1	-	+	+	-
G1349D	Y	NBD2	–	+	+	+[c]
A559T	Y	NBD1	-	+	–	-
A1357T	N	NBD2	–	+	+	+
D572N	N	NBD1	–	+	–	–
D1370N	N	NBD2	–	+	+	–
N894,900Q	N	ECD4[d]	+	–	–	+

[a] The known association with CF (Y, yes; N, no; P, polymorphism), domain location, presence (+) or absence (-) of CFTR bands A, B, and C, and presence (+) or absence (-) of function in the SPQ halide efflux assay are shown for each mutant CFTR species.

[b] The convention for naming mutants is as follows: the amino acid normally found at the particular residue, the residue number (25), and the amino acid to which the residue was converted. The single-letter amino acid code is used. Thus, K464M is a mutant in which lysine 464 is converted to methionine.

[c] Confirmation by whole-cell patch clamp.

[d] ECD, extracellular domain.

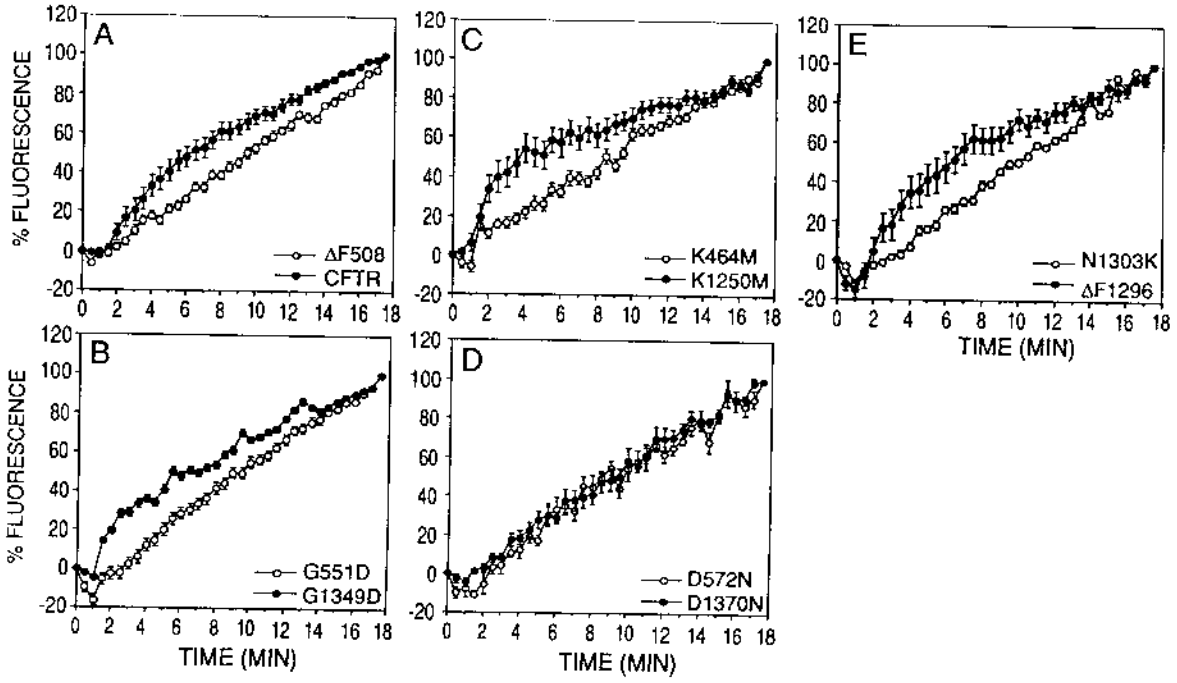


FIG. 3. Functional assay of CFTR mutants by SPQ fluorescence. The change in fluorescence of SPQ-loaded HeLa cells expressing wild-type or mutant CFTR is shown following substitution of NO_3^- for I^- in the bathing medium. Cells were stimulated with 20 μM forskolin and 100 μM isobutylmethyl xanthine for 7 min prior to ion substitution. Data are means ± standard errors of representative experiments for each mutant (CFTR, n = 33; ΔF508, n = 27; G551D, n = 40; G1349D, n = 52; K464M, n = 25; K1250M, n = 20; D572N, n = 6; D1370N, n = 7; N1303K, n = 40; ΔF1296, n = 18; where n = number of cells). Mutants were tested for function by using the SPQ assay with at least three different cell preparations. Values for each pair of mutants are significantly different ($P < 0.001$), except for the D-to-N pair of mutants, as assessed by Student's unpaired t test.

measured by the appearance of band C. In addition, the data extends our previous observation that cells transfected with CF-associated mutants commonly but not exclusively lack mature CFTR.

Functional assay for CFTR activity. To measure the function of the CFTR variants, we used the SPQ immunofluorescence assay in vaccinia virus-infected HeLa cells transfected with mutant plasmids (1, 24). Approximately 12 h after infection, SPQ-loaded cells were equilibrated in an NaI buffer. We then measured the increase in fluorescence of SPQ after the NaI buffer was replaced by $NaNO_3$ buffer (I^- quenches SPQ fluorescence but NO_3^- does not). The increase in fluorescence was measured in the presence and absence of cAMP. Representative data are shown in Fig. 3. We have previously shown that a cAMP-induced increase in the rate of fluorescence change results from activation of Cl^- channels (1, 24).

Table 1 summarizes the results. A number of the mutations, especially those in NBD2 (F508C, N894,900Q, K1250M, ΔF1296, S1347R, G1349D, and A1357T) retain cAMP-activated iodide efflux. The remainder (K464M, ΔI507, ΔF508, F508R, S5491, S549R, G551D, A559T, D572N and its NBD2 equivalent D1370N, and N1303K) lack activity. For mutations K1250M and G1349D, the SPQ data were confirmed by patch clamp measurements using the methods previously described (1, 10) (Fig. 4). In both cases, addition of cAMP reversibly stimulated Cl^- current.

The functional assay data are striking for two reasons. First, all CF-associated mutations, with the exception of G1349D, are negative in the functional assay. Second, mutations in NBD1 lead to functional inactivation more often than do the equivalent mutations in NBD2.

DISCUSSION

The absence of mature CFTR correlates with failure to activate the chloride channel. The data in Table 1 show that with the exception of the synthetic glycosylation site mutant N894,900Q, mutants that fail to make band C also fail to generate cAMP-activated Cl^- channels. We believe that this result is predictable since we earlier suggested that the absence of band C reflects the failure of some mutant CFTR species to traffic normally through the cell (3). We propose that such mutants are rejected in the endoplasmic reticulum by a cellular quality control mechanism. If this is the case, a mutant unable to make band C is one that is recognized by the quality control mechanism, and consequently cannot transport normally and cannot mature. Inevitably, such mutants will not progress to the plasma membrane and will lack functional activity at the cell surface.

Although all mutants except N894,900Q which lack the ability to make band C also lack functional activity, the corollary is not true; that is, all mutants associated with CF do not lack band C. We previously showed that G551D and the transmembrane 6 mutant R334W (3) both make apparently normal amounts of band C. Here we also demonstrate this for G1349D. This result means that not all mutations in CFTR result in effects that are recognized by the cellular quality control mechanism. In these cases, intracellular trafficking and maturation proceed normally, but nevertheless the protein is defective, presumably because a residue crucial to protein function is altered. It is also important to recognize that many mutations reported to be associated with CF are very rare, and in some cases insufficient evidence exists to prove formally that a given mutation

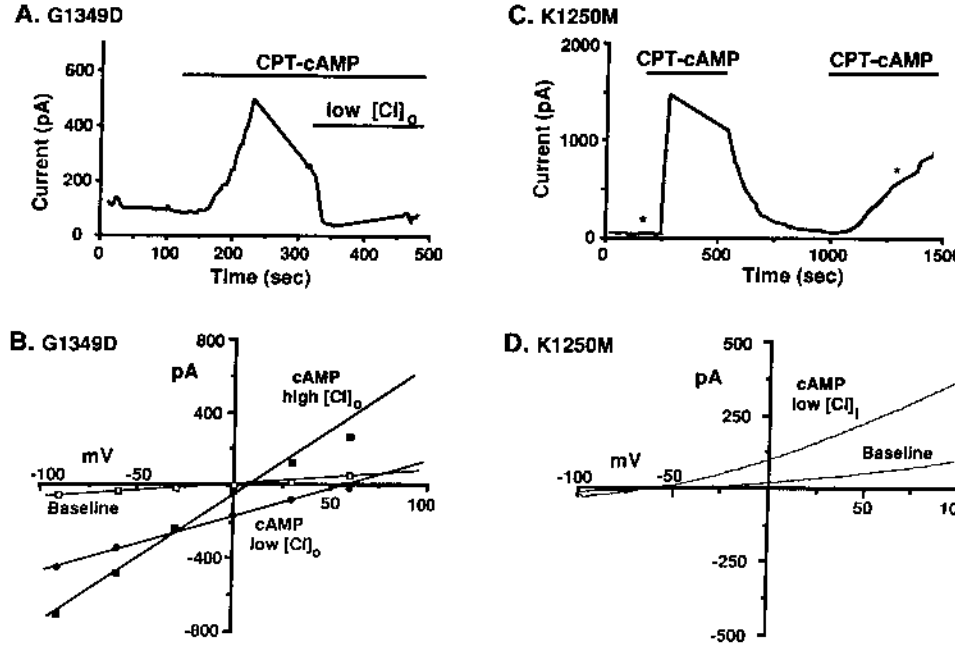


FIG. 4. Whole-cell Cl currents in CFTR mutants. Expression of CFTR NBD2 mutants G1349D and K1250M produces cAMP-activated Cl^- currents in HeLa cells. (A) Time course for activation of current in the G1349D CFTR mutant. Upper bar shows the time during which 8-(4-chlorophenylthio) (CPT)-cAMP (500 μM) was present. Lower bar shows the time when extracellular $[Cl^-]$ was decreased from 139.8 to 4.8 mM (low $[Cl]_o$). Values are currents recorded at +80 mV. (B) Current-voltage (I-V) relationship in the G1349D CFTR mutant obtained before (open symbols) and after (closed symbols) addition of CPT-cAMP (500 μM). Cell was the same as that shown in panel A. I-V relationships were obtained in symmetrical Cl solutions (■) and after reduction of the extracellular Cl^- concentration from 139.8 to 4.8 mM (●) (Cl^- replaced by aspartic acid; expected reversal potential, +84.8 mV). (C) Time course for activation and reversal of currents in the K1250M CFTR mutant; bar shows the times during which CPT-cAMP (500 μM) was present. (D) Current-voltage relationship of the currents shown in C. The I-V relationship was obtained from a continuous voltage ramp from −100 to +100 mV at the times indicated by the asterisk in panel C.

causes CF. G1349D is a case in point; this CFTR mutant is functional in our assays, suggesting the possibility that there are other, undiscovered mutations on the CF allele carrying G1349D and that these uncharacterized mutations cause CF.

Carbohydrate addition per se is not necessary for CFTR function. The data presented here indicate that even though inactive mutants commonly lack complete glycosylation, carbohydrate addition is not necessary for the function of CFTR. N894,900Q retains anion transport function even though it makes no band B or band C. We interpret this finding to mean that this mutant is able to traffic normally through the cell. This emphasizes that the quality control mechanism that detects other mutants does not recognize defective glycosylation, but rather lack of complete glycosylation is a consequence of rejection. We conclude that glycosylation is not essential for CFTR to get to its site of action or to generate cAMP-activated Cl^- channels. Other examples in which interference with the normal glycosylation of a transmembrane protein does not affect intracellular trafficking of that protein have been reported. For example, a deglycosylated version of the muscarinic acetylcholine receptor has been shown to be correctly localized and functional at the plasma membrane (30).

Abnormal chloride channel function does not prevent glycosylation of CFTR. The results with mutants that are inactive yet still produce band C provide evidence that defective function of CFTR does not prevent its own glycosylation. G551D is a naturally occurring CF mutation, and expression of G551D fails to increase cAMP-dependent halide permeability yet still produces band C. Similarly, D1370N, which affects a highly conserved aspartic acid in the Walker B motif of NBD2, lacks activity in the SPQ assay yet produces band C. We conclude that the absence of complete glycosylation of certain CFTR mutants does not result from lack of cAMP-activated Cl^- channel activity.

NBD1 is more susceptible than NBD2 to the effects of mutation. In the series of mutations presented in Table 1, it is clear that equivalent mutations in NBD1 and NBD2 do not have equivalent consequences in the assays used here. Eight of nine mutations in NBD1 (excluding the polymorphism F508C) but only one of seven mutations in NBD2 fail to make detectable band C. Likewise, all of the mutations in NBD1 tested (except F508C) abolish cAMP-stimulated anion permeability, but only two of seven mutations in NBD2 are inactive. Thus, mutations in NBD1 are at best equal to, but usually more severe than, the equivalent mutations in NBD2. Strikingly, these results reflect the observed uneven distribution of CF-associated mutations within CFTR. Of the 15 missense mutations shown in Fig. 1B, only 3 are found in NBD2 whereas 12 are present in NBD1. Thus, the molecular basis for the frequency of CF-associated mutations within the NBDs would seem to be the increased sensitivity of NBD1 mutations to the quality control mechanism or functional inactivation. We suggest that this difference reflects either the different sequences within the NBDs themselves or else differences in the adjacent domains with which they may interact. In this regard, it is notable that the sequence homology between NBD1 and NBD2 in CFTR is much less

than for other proteins of similar overall structure with duplicate NBDs. Interestingly, the results discussed above contrast with those of similar studies on the NBDs of MDR, in which equivalent mutations in the two domains generally have the same effect on function (2).

Given that mutations in the two NBDs affect maturation unequally, we believe that at least some of the differences in functional properties of mutants in the two domains are predictable. NBD1 mutants generally do not make band C; consequently, any potential partial activity of an NBD1 mutant is masked because it does not reach the correct cellular destination at which activity is measurable. The equivalent NBD2 mutants, by contrast, generally appear to escape the quality control mechanism and do make band C. Presumably, at least some of the NBD2 mutant CFTR is present at the plasma membrane, and this material, even though mutant, may in certain cases have measurable activity. Consider, for example, the paired mutations (K464M and K1250M) in the conserved lysine in Walker A, where K464M does not make band C and is functionally inactive whereas K1250M does make band C and retains functional activity. The differences observed do not necessarily mean that the functions of the two domains (say, in nucleotide binding) are different. Rather, residual activity of K464M may be masked by incorrect subcellular location, whereas partial activity of K1250M may be magnified by the non quantitative nature of the functional assay and expression system (see below). Similar arguments apply to other pairs of mutations.

Another factor that might emphasize differences in activity between NBD1 and NBD2 mutants is the expression system and functional assay. Of the mutants that make band C, we show that six of eight retain measurable anion transport function. However, because neither the SPQ assay nor the measurement of Cl^- currents with the patch clamp is completely quantitative (note our previous description of the heterogeneous response with use of the vaccinia virus hybrid expression system [1, 24]), we cannot conclude that functional mutants are completely wild type and retain 100% activity. Indeed, the apparent full activity of ΔF1296, which in COS-7 cells makes much less band C than does the wild type, and of G1349D, which is reported to be associated with CF, suggests that in some cases our expression system and functional assay may not allow discrimination of partially functional from fully functional mutants. It is also possible that the high level of expression of CFTR in the HeLa and COS-7 cells might influence the assays to mask some functional defects.

What is the function of the NBDs? The results presented above do not allow us to unambiguously ascertain the biochemical properties of the NBDs. However, very recently Thomas et al. (29) showed that a synthetic peptide including the Walker A motif of NBD1 interacts with adenine nucleotides. Assuming, as seems probably, that both conserved Walker motifs are associated with nucleotide binding, it is important to note that two steps are likely involved: first, the binding or hydrolysis of nucleotide and second, the functional consequences that result from nucleotide binding or hydrolysis. If this reasoning is correct, even if NBD1 and NBD2 of CFTR both bind nucleotide, the functional consequences for CFTR that result from that binding may be different. Several arguments favor a different role for the two domains resulting from the different consequences of nucleotide binding. These include the clustering of CF-associated mutations and the differential effects reported here of seemingly equivalent mutations both on maturation of CFTR and on Cl^- channel activity. However, the fact that certain mutations in NBD2 do affect CFTR maturation or Cl^- channel activity suggests that the two NBDs may play similar roles in CFTR function and that the primary difference between the two NBDs is the greater susceptibility of NBD1 mutants to detection by the quality control mechanism. The resolution of these alternative possibilities requires further study.

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