A Mutation in the Cystic Fibrosis Transmembrane Conductance Regulator Generates a Novel Internalization Sequence and Enhances Endocytic Rates*

Received for publication, December 17, 2002, and in revised form, January 14, 2003
Published, JBC Papers in Press, January 15, 2003, DOI 10.1074/jbc.M212843200

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Cystic fibrosis is a common lethal genetic disease among Caucasians. The cystic fibrosis gene encodes a cyclic adenosine monophosphate-activated chloride channel (cystic fibrosis transmembrane conductance regulator (CFTR)) that mediates electrolyte transport across the luminal surfaces of a variety of epithelial cells. Mutations in CFTR fall into two broad categories; those that affect protein biosynthesis/stability and traffic to the cell surface and those that cause altered channel kinetics in proteins that reach the cell surface. Here we report a novel mechanism by which mutations in CFTR give rise to disease. N287Y, a mutation within an intracellular loop of CFTR, increases channel endocytosis from the cell surface without affecting either biosynthesis or channel gating. The sole consequence of this novel mutation is to generate a novel tyrosine-based endocytic sequence within an intracellular loop in CFTR leading to increased removal from the cell surface and a reduction in the steady-state level of CFTR at the cell surface.

Disruption of intracellular trafficking events has been recognized as the underlying molecular basis for a growing number of human genetic diseases including diabetes mellitus, familial hypercholesterolemia, Hermansky-Pudlak syndrome, α1-antitrypsin activity, and cystic fibrosis (CF)1 (1). Cystic fibrosis, a common lethal autosomal recessive genetic disease, results from mutations in the CF transmembrane conductance regulator (CFTR) and affects ~1 in 2,000 live births in Caucasians (2–4). CFTR, a member of the ATP-binding cassette transporter family of proteins (3), functions as a cAMP-activated anion channel and channel regulator that resides at the apical plasma membrane of polarized epithelia where it regulates epithelial secretions in the respiratory and gastrointestinal tracts (5–7). Cystic fibrosis is characterized by high sweat chloride levels, pulmonary disease, and pancreatic insufficiency, although other organs including kidney, liver, and tissues of the reproductive tract are also affected (2, 4). The most common disease-causing mutation in CFTR is a 3-base pair deletion, resulting in the loss of a phenylalanine residue at position 508 (ΔF508) (3). This mutation accounts for ~70% of all mutant alleles and generates a protein that is unable to fold appropriately. Misfolded ΔF508 CFTR is retained within the endoplasmic reticulum (ER) and eventually degraded by the proteasome. Although the molecular basis for disease in patients bearing the ΔF508 mutation has been extensively studied, little is known about the molecular bases for disease in the more than 1000 different mutations that have been detected in CF patients (Cystic Fibrosis Genetic Analysis Consortium, www.genet.sickkids.on.ca/CFTR).

In addition to its localization within the apical plasma membrane, morphological, biochemical, and functional data indicate that CFTR is also localized to endosomal and recycling compartments (8–11). The distribution of CFTR between endosomes and the plasma membrane appears to be in a dynamic equilibrium. The rapid efficient endocytosis of CFTR is mediated by clathrin-coated pits in both polarized and non-polarized cells, and CFTR can be detected in isolated clathrin-coated vesicles (9). Efficient internalization of integral membrane proteins relies on the presence of short peptide sequences within their cytoplasmic domains. Tyrosine- and dileucine/leucine-based endocytic signals have been the most extensively studied endocytic signals identified in type I and type II membrane proteins (12, 13). Studies utilizing full-length (14) and chimeric proteins fused to either the transferrin receptor (15) or the interleukin 2 receptor (16) have identified a tyrosine-based motif1424YDSI in the carboxyl tail of CFTR. Moreover, cross-linking and in vitro pull-down assays demonstrated that Tyr1424 associates with the μ subunit of the AP-2 component of the clathrin endocytic machinery (17). Recently an individual with mutation N287Y (991A→T) was identified based on a diagnosis of elevated sweat electrolytes (18). Since tyrosine-based signals are important in endocytic targeting, we hypothesized that the N287Y mutation generated a novel additional internalization signal in CFTR (Fig. 1a), leading to reduced cell surface expression of CFTR as a result of increased endocytic activity. Using site-directed mutagenesis in conjunction with morphological, biochemical, and functional assays, we demonstrate that N287Y CFTR generates a novel endocytic sequence enhancing the endocytic rate of CFTR compared with wild type. Such increased endocytosis leads to a reduced steady-state level of CFTR at the plasma membrane, likely accounting for the CF phenotype observed in patients bearing this mutation. Moreover, since neither biosynthesis nor single channel kinet-
ics are appreciably altered in this mutation, the sole molecular basis for this disease-causing mutation appears to be altered endocytic rates. These studies reveal not only a novel class of CFTR mutations but also a new paradigm in the localization of endocytic sequences in polytopic proteins.

**EXPERIMENTAL PROCEDURES**

**Construction of CFTR Mutants**—Full-length human CFTR cDNA from pBQ4.7 was subcloned into the pcDNA5/FRT vector (Invitrogen). An NheI site was introduced (QuikChange Site-Directed Mutagenesis kit, Stratagene) upstream of the CFTR coding region, and CFTR was subcloned into pcDNA5/FRT using restriction site NheI and a pre-existing carboxyl-terminal XhoI site into the multiple cloning site within the pcDNA5/FRT vector. The N287Y mutation was introduced into the pFRT CFTR wild-type vector using the QuikChange Site-Directed mutagenesis kit (Stratagene). The sequences of the pcDNA5/FRT-CFTR wild type and N287Y were verified prior to use for expression.

**Cell Lines and Transfections**—293 and CHO Flp-In724 (Invitrogen) isogenic cell lines expressing wild-type, ΔF508, and N287Y CFTR from the same genomic locus were generated according to the manufacturer’s instructions. Confirmation of expression was determined by immunofluorescence microscopy, immunoblot, and immunoprecipitation. All cells were grown at 37°C in 5% CO₂ under standard conditions. Confluent monolayers of Madin-Darby canine kidney cells (type II) grown on permeable filter supports were transiently transfected with either wild-type or N287Y CFTR using calcium phosphate. Seventy-two hours later, stimulated with 10 μM forskolin and 100 μM cAMP, the patch pipette solution was replaced with complete minimum Eagle’s medium supplemented with 10 mM HEPES (pH 8.0), 3 mM KC1, 1 mM MgCl₂, and 0.5 mM CaCl₂) at 4°C. Cells were washed in ice-cold phosphate-buffered saline supplemented with bovine serum albumin (0.1%) and lysed. Biotinylated CFTR was isolated by precipitation on streptavidin-Sepharose (Sigma). Precipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody (M3A7) against CFTR. Biotinylated CFTR was visualized by enhanced chemiluminescence and quantified by densitometric analysis. To quantify cell surface insertion of CFTR, cells metabolically labeled using a pulse-chase protocol were biotinylated during the subsequent 1-h chase period. Cells were washed with ice-cold phosphate-buffered saline supplemented with 0.1% bovine serum albumin, 1 mM MgCl₂, and 0.1 mM CaCl₂ and lysed. Biotinylated CFTR was isolated by immunoprecipitation with M3A7 anti-CFTR antibodies and then on streptavidin-Sepharose (Sigma). Biotinylated CFTR was visualized with fluorography, and radioactivity was measured by phosphorimage analysis.

**Patch Clamp Analysis**—Whole cell and cell-attached patch clamp studies were performed on CHO Flp-In cells expressing wt CFTR or N287Y CFTR. The patch pipette solution for whole cell studies contained 100 mM l-aspartic acid, 100 mM CsOH, 40 mM CsCl, 1 mM NaCl, 1 mM EGTA and 10 mM TES (pH 7.2). One millimolar Mg-ATP and 50 μM Mg-GTP were added to the pipette solution before each experiment. The bath solution contained 140 mM NaCl, 4 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 30 mM mannitol, and 10 mM TES (pH 7.4). Mannitol was used to avoid the stimulation of swelling-activated chloride channels. The mannitol was eliminated from the bath solution for the cell-at- tached patch clamp studies, and this same solution was used in the patch pipette. All experiments were performed at 37°C. Cells were stimulated with 10 μM forskolin and 100 μM cAMP added to the bath perfusate. Current-voltage curves were constructed using voltage pulses of 250-ms duration over a range of −70 to +70 mV at 10-mV increments. Data acquisition and analysis were performed using pClamp software (version 8.0, Axon Instruments). Values for P₀ were obtained from amplitude histograms of multichannel patches. Channel records of at least 3-min duration were evaluated. The maximum current amplitude was noted and divided by the single channel amplitude to obtain an estimate of N for each cell.

**RESULTS**

ER Export and Polarity of CFTR Distribution Is Preserved in N287Y CFTR—The cellular distribution of N287Y CFTR was initially examined since intracellular retention of mutant CFTRs by the ER quality control and subsequent failure to mature to a complex-glycosylated form is the most prevalent form of CF. In contrast to ΔF508 CFTR, which displayed an ER-like perinuclear distribution pattern (Fig. 1c), both wild-
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**Fig. 2.** Polarized distribution of wild-type and N287Y CFTR in Madin-Darby canine kidney cells. Confocal micrograph of Madin-Darby canine kidney II cells transiently expressing wild-type and N287Y CFTR. CFTR fluorescence is green (M3A7 and L12B4, Upstate Biotechnology), and ZO-1 (Zymed Laboratories Inc., San Francisco, CA), a protein in tight junctions that separates apical and basolateral membrane domains, is red. Images show confocal sections taken at the plane of the apical membrane (a and b), plane of the tight junction (c and d), and plane of the basal membrane (e and f) for wild-type (a, c, and e) and N287Y (b, d, and f) CFTR. g, immunoblot of cells expressing wild-type and N287Y CFTR following domain-selective cell surface biotinylation. Mature, fully glycosylated CFTR band C is ~190 kDa. Ap, apical membrane; Bl, basolateral membrane.

**Fig. 3.** Steady-state levels and cell surface expression of wild-type and N287Y CFTR. a, expression level of total and cell surface CFTR. Equal amounts of metabolically labeled 293 cells expressing wild-type or N287Y CFTR were subject to immunoprecipitation using the M3A7 anti-CFTR antibody followed by phosphorimage analysis (top). Plasma membrane proteins were biotinylated with 1 mg/ml sulfo-NHS-SS-biotin for 60 min at 4 °C and isolated on streptavidin-Sepharose, and the precipitate was subjected to immunoblot analysis using M3A7 anti-CFTR antibodies (bottom). The sulfo-NHS-SS-biotin remains membrane-impermeant during the labeling as shown by the lack of biotinylated core-glycosylated forms. Complex- and core-glycosylated forms are indicated by black and white arrows, respectively. b, the expression level of wt and N287Y CFTR at the cell surface (biotinylated) and post-ER compartments (complex-glycosylated). Data represent means ± S.E. (n = 3) and were normalized by expressing the data as a percentage of wild type, designated as 100%. c, glycosidase sensitivity of wild-type and N287Y CFTR. Cell lysates were incubated in the presence or absence of endoglycosidase H (15 milliunits of endo H) or peptide-N-glycosidase F (250 milliunits of PNGase F) for 3 h at 37 °C. Proteins were resolved by SDS-PAGE and probed with M3A7 anti-CFTR antibodies. Complex-, core-, and deglycosylated CFTR are indicated by black, white, and gray arrowheads, respectively.

type and N287Y CFTR were seen at the cell surface (Fig. 1, b and d). Similar results were obtained for N287Y CFTR stably expressed in CHO cells (data not shown). These results suggest that intracellular retention is unlikely to account for the clinical phenotype associated with N287Y CFTR. To determine whether the N287Y mutation altered the polarization of CFTR in epithelial cells, we transiently expressed wild-type and N287Y CFTR in Madin-Darby canine kidney cells. Confocal immunofluorescence microscopy demonstrated that wild-type CFTR was polarized to the apical plasma membrane (Fig. 2a) with little or no staining of the basal membrane (Fig. 2e). The ratio of wild-type CFTR in the apical membrane versus the basolateral membrane was 10.6 ± 2.9 as determined by domain-selective cell surface biotinylation (Fig. 2g). Similarly N287Y CFTR was also polarized to the apical plasma membrane (Fig. 2b). The ratio of N287Y CFTR in the apical membrane relative to the basolateral membrane was 8.1 ± 3.1 as determined by domain-selective cell surface biotinylation.
Thus, abnormalities in the polarized distribution of N287Y CFTR compared with wild-type CFTR cannot account for the disease phenotype associated with this mutation.

**N287Y CFTR Shows Altered Cellular Distribution**—Quantitative immunoblot analysis of whole cell lysates from isogenic wild-type and N287Y CFTR-expressing cells revealed that steady-state levels of protein expression were identical in each cell line (Fig. 3, a and b). Moreover, the ratio of fully glycosylated mature band C CFTR to immature core-glycosylated band B CFTR was not altered by the N287Y mutation compared with wild-type CFTR (7.2 ± 0.5 and 6.9 ± 0.5, mean ± S.E., n = 4 for wild-type and N287Y CFTR, respectively). In addition, the complex- and core-glycosylated forms of wild-type and N287Y CFTR could be distinguished by their sensitivity to endoglycosidases, which was not different between the two cell lines (Fig. 3c). To evaluate the amount of CFTR present at the plasma membrane, cell surface biotinylation experiments were performed. In both wild-type and N287Y CFTR-expressing cell lines, biotinylated CFTR was detected as a single band of ~170 kDa, consistent with the presence of mature fully glycosylated CFTR at the cell surface. However, densitometric analysis revealed that the level of biotinylated N287Y CFTR was only ~50% of that for wild-type CFTR (Fig. 3, a and b). The detection of the mature complex-glycosylated form of wild-type and N287Y CFTR in immunoblots and at the cell surface demonstrates that biosynthesis and intracellular transport occurred.

**Biosynthesis of N287Y CFTR Is Not Impaired**—To better evaluate the efficiency of N287Y CFTR biosynthesis, pulse-chase experiments were performed (Fig. 4, a and b). Immediately following the pulse, the entire immunoprecipitated label was associated with a broad band migrating at ~140 kDa. This band was identified as ER-associated core-glycosylated CFTR based on the following criteria. First, its electrophoretic mobility corresponds to that previously observed (for band B) in other CFTR-expressing cell lines (20, 21). Second, it was immunoprecipitated with several monoclonal antibodies against CFTR but not with an unrelated control antibody (data not shown). Third, it was detected only in 293 cells stably trans-
fected with CFTR cDNA and not in “parental” 293 Flp™ cells (21). Band B CFTR intensity decreased during the chase, concomitant with the appearance of a slower migrating species (~160–180 kDa; Fig. 4, a and b) corresponding to mature fully glycosylated CFTR (band C). Following a 30-min pulse, labeling of band B decreased rapidly over 60 min with mature band C CFTR first becoming detectable at 30 min of chase and reaching a plateau by 120 min. Labeling of band C CFTR did not show any evidence of decline during chase periods up to 480 min, consistent with observations that band C CFTR is considerably more stable than band B CFTR. As reported previously, the conversion of the wild-type band B CFTR to band C was not very efficient, reaching a maximum of ~40% after ~2 h of chase (with the remaining 60% of synthesized band B being degraded). The apparent normal biosynthesis of mature fully glycosylated N287Y CFTR suggests that targeting to the plasma membrane is largely intact. Wild-type and N287Y CFTR were pulse-labeled with [35S]methionine, and those molecules that arrived at the cell surface were biotinylated throughout the subsequent chase. Biotinylated CFTR was affinity-isolated and visualized by fluorography (Fig. 4, c and d). The cell surface targeting efficiency of N287Y CFTR was 91 ± 4% (mean ± S.E., n = 3) of wild-type CFTR, suggesting that biosynthesis and plasma membrane delivery of N287Y CFTR is largely uncompromised. The turnover of complex-glycosylated wild-type and N287Y CFTR was assessed by pulse-chase labeling. As previously shown (21, 22), the t½ of stably expressed complex-glycosylated wild-type CFTR was ~12–14 h (Fig. 4c). The stability of complex-glycosylated N287Y CFTR stably expressed in 293 cells was not significantly different from that observed for wild-type CFTR.

N287Y CFTR Displays Abnormal Endocytic Trafficking—Since biosynthesis and membrane insertion of N287Y CFTR was uncompromised, we hypothesized that the reduction in cell surface N287Y CFTR compared with the wild type was likely due to an increase in endocytic retrieval from the plasma membrane. Endocytic internalization was determined by detecting the extent to which cell surface biotinylated CFTR was endocytosed and refractory to subsequent removal of remaining cell surface biotin following exposure to membrane impermeant thiol-reducing agents (19). Direct evidence for enhanced endocytosis of N287Y CFTR was obtained by determining the amount of thiol-resistant biotinylated CFTR with respect to time. The rapid increase in thiol-resistant biotinylated wild-type and N287Y CFTR indicates that both constructs are internalized with high efficiency (Fig. 5). Approximately 32% of wild-type CFTR was endocytosed during the first 5 min of incubation, showing an internalization rate of 6.6 ± 0.5%/min (n = 4), a rate similar to that reported previously (15–17). In contrast, the internalization rate of N287Y CFTR was nearly 2-fold faster (12.4 ± 0.4%/min, n = 4), implying that the tyrosine substitution enhanced the endocytic activity of CFTR.

Channel Density, but Not Single Channel Properties, Is Altered in N287Y CFTR-expressing Cells—We have documented abnormalities in the endocytic, but not biosynthetic, traffic of N287Y CFTR that result in a decrease in biotinylatable CFTR at the cell surface. We propose that this reduction is sufficient to lead to the mild clinical phenotype observed in this patient. However, it is formally possible that there are also alterations in the biophysical fingerprint of N287Y CFTR compared with wild type. To confirm that N287Y CFTR is functional at the plasma membrane, electrophysiological recordings were performed in the whole cell and cell-attached patch configurations on CHO cells stably expressing either wild-type or N287Y CFTR. Whole cell measurements were used to obtain an estimate of channel density. Expression of both wild-type and

**Fig. 5. Internalization efficiency of CFTR is increased by the N287Y mutation.** The rate of removal of CFTR from the cell surface was monitored as an increase in biotinylated CFTR resistant to thiol cleavage of the biotin moiety as described under “Experimental Procedures.” Cells stably expressing wild-type (filled circle) or N287Y (open circle) CFTR were subjected to cell surface biotinylation at 4 °C, washed, and left to internalize CFTR at 37 °C. Biotin remaining at the cell surface was removed using a membrane-impermeant reducing agent. Cells were lysed, biotinylated proteins were isolated on streptavidin beads, and CFTR was detected by immunoblot analysis. Results are means ± S.E. for four separate experiments.

N287Y CFTR conferred cAMP-stimulated whole cell currents in CHO cells (Fig. 6) with no change in base-line current in the absence of cAMP. The cAMP-stimulated whole cell conductance was 2.6-fold higher in wt CFTR-expressing cells compared with N287Y CFTR-expressing cells. Cell-attached patch studies revealed the single channel properties of the N287Y CFTR were nearly identical to that of wt CFTR. The single channel conductance of N287Y CFTR was 9.7 ± 0.35 versus 9.8 ± 0.39 picosiemens and open probabilities were 0.48 ± 0.04 versus 0.46 ± 0.05 for N287Y CFTR and wt CFTR, respectively. These results strongly support the conclusion that the lower whole cell conductance of the N287Y CFTR-expressing cells is the result of a lower density of functional channels in the plasma membrane.

**DISCUSSION**

We have demonstrated that the N287Y mutation in CFTR causes clinical disease by dramatically increasing the rate at which CFTR is sequestered from the plasma membrane without altering its channel properties. Although there are several classes of CF-causing mutations, they nonetheless can be broadly grouped into two main categories (23, 24). The first group includes those mutations that affect channel density at the cell surface either by affecting protein synthesis (class I); by reducing protein folding, processing, and export from the endoplasmic reticulum to the plasma membrane (class II); or by altering stability and abundance of mRNA or protein (class V). Disease-associated mutations within the second group generate CFTR molecules that reach the cell surface but fail to appropriately transport chloride ions either because of a defect in regulation by ATP and protein kinase A (class III) or channel conductance (class IV). Several mutations, including the ΔF508 mutation, display disruption in both protein traffic and ion channel activation/conductance. Recently Lukacs and colleagues (22) have suggested a class VI mutation that would alter CFTR molecules that reach the cell surface but fail to appropriately transport chloride ions either because of a defect in regulation by ATP and protein kinase A (class III) or channel conductance (class IV). Several mutations, including the ΔF508 mutation, display disruption in both protein traffic and ion channel activation/conductance. Recently Lukacs and colleagues (22) have suggested a class VI mutation that would include mutations in CFTR that alter protein stability within the plasma membrane by increasing degradation rates. Several point mutations have been described in the amino-terminal cytoplasmic loops of CFTR in CF patients (25, 26). Based upon these studies and others (27), the intracellular loops of CFTR are emerging as significant contributors to CFTR trafficking and function. Approximately half of the disease-causing point mutations identified in the second intracellular loop give rise to processing mutants; the rest appear to process appropriately but have significantly reduced open probabilities.

Here we report a novel class of CFTR mutation. In contrast
to previously reported mutations in CFTR, the biosynthesis, exocytic insertion into the plasma membrane, overall protein stability, and single channel kinetics are unaltered; only endocytic kinetics are changed. The N287Y mutation, a mutation in the second intracellular loop, results in a channel that is biosynthetically and biophysically normal but has greater endocytosis kinetics compared with wild-type CFTR. This increased endocytic rate results in a significant reduction (~50%) in steady-state CFTR levels in the plasma membrane as determined by cell surface biotinylation and whole cell patch clamp analysis. Similarly targeting of CFTR to the apical plasma membrane domain of polarized epithelial cells is unaffected by the N287Y mutation. In contrast to other mutations in the second intracellular loop, the N287Y mutation has no functional consequence on the gating kinetics of CFTR. Thus open probability and single channel conductance are essentially wild type. The only physiological consequence of the N287Y mutation is therefore to produce a more rapidly endocytosed protein, reducing steady-state levels in the plasma membrane. The genotype of the initial patient reported to have the N287Y mutation was ΔF508/N287Y (18). Since little or no cell surface CFTR is produced by the ΔF508 allele, the only cell surface CFTR protein is produced by the N287Y allele. This would lead to a 75% reduction in the amount of CFTR at the cell surface in this patient compared with an individual homozygous for wild-type CFTR.

The most parsimonious interpretation of our data is that the N287Y mutation generates a novel tyrosine-based endocytic motif. Yet the mutant tyrosine-containing sequence (284MIE-LRQT) does not conform to the canonical NPXY or YXXΦ sequences where X is a variable amino acid and Φ is a bulky hydrophobic. However, non-canonical tyrosine-based endocytic motifs have recently been described in ionotropic receptors (28) that result in AP-2-mediated targeting to the clathrin-dependent endocytic pathway. Thus it is possible that the N287Y mutation results in the generation of a sequence that displays modest affinity for the AP-2 clathrin adaptor complex. Although we have focused upon the role of the N287Y mutation in mediating enhanced endocytosis of CFTR, it is still a formal possibility that the N287Y mutation could also reduce the endocytic recycling rate. However, it still remains to be determined whether CFTR does indeed recycle or whether endocytosed CFTR is degraded. It is of interest to note that the N287Y mutation is a gain of function mutation and appears to generate an endocytic signal that is present within the body of the protein rather than at the termini of the protein, a localization not previously identified in polytopic membrane proteins. The first identification of an endocytic targeting sequence and a disease-causing mutation within that sequence was the NPVY sequence identified in the cytoplasmic tail of the low density lipoprotein receptor; mutations in this sequence result in an inhibition of endocytic internalization of the low density lipoprotein receptor (29) with concomitant increases in plasma low density lipoprotein. In contrast, our studies report a mutation that generates an additional endocytic signal resulting in increased rates of endocytosis and loss of CFTR from the cell surface. In the broader context of molecular mechanisms underlying the pathology of human genetic diseases, the significance of our observations lies in the recognition that mutations can reduce the expression level of a membrane protein not only by impairing its biosynthesis or stability (or in the case of ion channels their biophysical fingerprint) but also by accelerating endocytic retrieval from the plasma membrane.

Acknowledgments—We thank S. W. Watkins for help with immunofluorescence imaging and analysis and R. A. Frizzell for critical review of the manuscript.

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