C-terminal Truncations Destabilize the Cystic Fibrosis Transmembrane Conductance Regulator without Impairing Its Biogenesis

**A NOVEL CLASS OF MUTATION**

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Defective cAMP-stimulated chloride conductance of the plasma membrane of epithelial cell is the hallmark of cystic fibrosis (CF) and results from mutations in the cystic fibrosis transmembrane conductance regulator, CFTR. In the majority of CF patients, mutations in the CFTR lead to its misfolding and premature degradation at the endoplasmic reticulum (ER). Other mutations impair the cAMP-dependent activation or the ion conductance of CFTR chloride channel. In the present work we identify a novel mechanism leading to reduced expression of CFTR at the cell surface, caused by C-terminal truncations. The phenotype of C-terminally truncated CFTR, representing naturally occurring premature termination and frameshift mutations, were examined in transient and stable heterologous expression systems. Whereas the biosynthesis, processing, and macroscopic chloride channel function of truncated CFTRs are essentially normal, the degradation rate of the mature, complex-glycosylated form is 5- to 6-fold faster than the wild type CFTR. These experiments suggest that the C terminus has a central role in maintaining the metabolic stability of the complex-glycosylated CFTR following its exit from the ER and provide a plausible explanation for the severe phenotype of CF patients harboring C-terminal truncations.

Cystic fibrosis (CF) is the most prevalent genetic disease in the Caucasian population and manifests in pleiotropic defects on the physiology of epithelia lining the lung, gastrointestinal tract, reproductive organs, and sweat duct (1–3). The CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride selective ion channel, consists of two structurally homologous halves connected by the regulatory domain, wherein each half contains a transmembrane domain, comprising six transmembrane helices, and a nucleotide binding domain (NBD) (2). Activation of the CFTR chloride channel requires the phosphorylation of the regulatory domain by cAMP-dependent protein kinase A (PKA) and hydrolysis of ATP at the NBDs (4–6). The severity of the CF phenotype correlates with the severity of the defect in the cAMP-stimulated chloride conductance at the apical membranes of afflicted epithelia (4, 7, 8). Most of the CF-associated point mutations, located in the NBDs and the regulatory and transmembrane domains or in the cytosolic loops, are thought to interfere with the biosynthesis, processing, or functioning of CFTR, leading to an impaired anion conductance of the plasma membrane (8–13). In contrast, the molecular phenotype of CFTR variants harboring mutations in the C-terminal tail has not been extensively investigated. Interestingly, patients with a premature stop codon or frameshift mutation that causes the deletion of the last 70–98 residues have severe CF with pancreatic insufficiency, recurrent lung infections, and elevated sweat chloride (Cystic Fibrosis Genetic Consortium Database, Toronto and footnote 2), suggesting that the C-terminal tail may play a role in the function of CFTR.

In this study, we have examined the biochemical and functional characteristics of CFTR mutants caused by premature terminations. Although the C-terminal tail is not required for the biogenesis and macroscopic chloride channel function of CFTR, it appears indispensable for maintaining the stability of the complex-glycosylated CFTR. Therefore, our results highlight a previously unrecognized role of the C terminus and describe a novel mechanism of CF that is determined by the accelerated degradation of the mature, truncated CFTR.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The following compound heterozygote patients were identified in the CF Genetic Consortium Database, with a ΔF508 mutation and a premature stop codon or frameshift mutation in the second allele. Frameshift mutations were as follows: 4326delTC (M. Goosens and J. Zielenski; Δ81, +10), 4279insA (A. Wallace; Δ97, +1), 4271delC (S. J. Shackleton; Δ101, +3). Premature stop codons were as follows: Q1412X (A. Wallace and M. Tassabehji; Δ70); S1455X and L1399X (Δ26 and Δ82, respectively). The communicating person, the number of residues deleted from the C terminus of CFTR, and the

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1 The abbreviations used are: CF, cystic fibrosis; ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide binding domain; PKA, protein kinase A; WT, wild type; mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle’s medium; IBMX, 3-isobutylmethyl-1-xanthine; CTP, 8-(4-chlorophenylthio); HA, hemagglutinin; NHERF, Na+/H+ exchanger regulatory factor.

C. Taylor (University of Sheffield, UK), personal communication.
number of residues changed between the frameshift mutation and the premature stop codon are indicated. Premature stop codons were inserted by site-directed mutagenesis, using single-stranded pBluescript SKII-CFTR as the template (kindly provided by Dr. J. Rommens), by the method of Kunkel (15) to obtain CFTR lacking the last 26, 70, 82, and 98 amino acid residues. For T26 and T82 CFTR, the mutagenic primers (5′-CCC CAC CGG AAC TGA AGC AAG TGC-3′ and 5′-GCT GAT TCG ACA GTA ATT TGA CTC TGT GAA CAC AGG-3′ (ACGT

expression of T70 CFTR. Constructs were confirmed by DNA sequencing using the dideoxy termination method with T7 DNA polymerase.

Expression of Mutant and WT CFTR—To avoid the possible impact of clonal variations on CFTR trafficking, experiments were carried out both in transient and stable heterologous expression systems. COS-1 cells were transiently transfected with the pCDNA3 plasmid (Invitrogen) containing wild type (WT) or mutant CFTR cDNA, using LipofectAMINE. Stable transfectants of BHK-21 cells, expressing the WT and truncated CFTRs, were generated using the pHUT expression plasmid with methotrexate (500 μg/ml) selection (15). 12–24 individual clones were isolated and screened by Western blotting with mAb M3A7 (16). At least two to three representative clones were used for biochemical and electrophysiological studies. In addition, expression level and initial rate of biosynthesis of mutant CFTRs were determined in the mixture of BHK-21 clones after 2 weeks of selection. Western blotting with the mAbs L12B4, M3A7 (16), and 24-1 (Genzyme Inc. (17)) and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) were performed as described (18). The epitopes of mAb L12B4 and mAb M3A7 are located within the range of amino acid positions 386–412 and

A

control

stimulated

B

membrane potential (mV)

T70

T26

T98

current (pA)

wt

C

membrane potential (mV)

D

current density (pA/μF)

BHK

WT

T26

T70

T98

WT (50/140)

+ cAMP

T26 (50/140)

T70 (50/140)

T98 (50/140)

(7)

(8)

(9)

(6)

(10)

(11)

(12)

(13)

FIG. 1. Immunofluorescence localization of wild type (wt) and truncated CFTR constructs. COS-1 cells were transfected with WT or truncated CFTR constructs bearing an N-terminal influenza HA epitope. CFTR was visualized by immunostaining with monoclonal anti-HA antibody and fluorescein-conjugated anti-mouse secondary antibody. Photographs were taken on a Zeiss Axiovert 100 inverted fluorescence microscope, using a Planachromat 63x/1.35 objective. For comparison, immunostaining of ΔF508 CFTR is shown as an inset.

FIG. 2. Electrophysiological characterization of the truncated CFTR constructs. A, whole cell current recordings from BHK-21 cells expressing WT, T26, T70, T82, and T98 CFTR before (control) and after stimulation with 20 μM forskolin, 0.2 mM IBMX, and 0.5 mM CTP-cAMP. B, the current-voltage relationship was determined before (filled square) and after (empty symbols) activation of PKA in BHK-21 cells expressing WT (squares and diamonds) or T26 CFTR (circles and triangles). For WT CFTR, current-voltage curves were recorded under asymmetrical and symmetrical conditions as indicated. C, PKA-stimulated current-voltage characteristics of BHK-21 cells expressing T70, T82, and T98 CFTR (empty squares, triangles, and circles, respectively) under asymmetrical conditions, as described in B, and for T98 CFTR under symmetrical conditions (empty inverted triangles). The current-voltage relationship for T70 CFTR is depicted before activation as well (filled squares). D, comparison of the stimulated whole cell current densities of BHK-21 cells expressing WT and truncated CFTR constructs. Maximally stimulated whole cell currents were measured before (control) and after stimulation at 75 mV of holding potential, normalized for cellular capacitance. For comparison, the current density of CHO-BQ1 cells, expressing ~10% of WT CFTR found in BHK-21 cells, is shown.
Accelerated Turn-over of the Mature CFTR

3.8–19.5% fetal bovine serum for the specified time. Cells were solubilized in DMEM (COS-1 cells) or F12/DMEM (BHK-21 cells) supplemented with 5–10% fetal bovine serum for the specified time. Cells were solubilized in RIPA buffer (150 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate, pH 8.0) containing 10 μg/ml each of leupeptin and pepstatin, 10 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride, and CFTR was immunoprecipitated with mAbs Li2B4 and M3A7. The radioactivity associated with CFTR was quantified using a PhosphorImager (PD1) and ImageQuant software.

Electrophysiology—Whole cell currents were recorded by the method of Hamill et al. (19). The pipette filling solution contained (in mM) 110 sodium gluconate, 20 NaCl, 8 MgCl2, 5 EGTA, 10 glucose, 2 ATP, and 10 HEPES, pH 7.2. The bath solution was as follows: 137 NaCl, 3 MgCl2, 1 CaCl2, 10 glucose, 10 HEPES, pH 7.2. Where indicated, gluconate substitution was used to lower the extracellular Cl concentration to 36 mM. The holding potential was set to –60 mV. For an analysis of the current-voltage relationship, the potential was stepped between –90 and +90 mV in 15-mV increments. Voltage steps were applied for 300 ms at 800-ms intervals. Currents were recorded and analyzed using the AXOPATCH and CLAMPFIT software, respectively (20). Currents were normalized per unit capacitance to account for variations in cell size. All electrophysiological measurements were conducted at room temperature (22–24 °C).

Immunolocalization—Indirect immunofluorescence localization of WT and truncated CFTR (N-terminally tagged with the influenza hemagglutinin (HA) epitope) was performed in COS-1 cells. After 36 h of transfection by calcium-phosphate precipitation, cells were exposed to 4 mM sodium butyrate overnight to enhance the expression of the transgene. HA-tagged CFTRs were visualized with murine monoclonal anti-HA antibody (Jackson Immunoresearch Laboratory Inc.) and fluorescein-conjugated donkey anti-mouse antibody. In contrast to F508 CFTR, open arrowheads, core-glycosylated CFTR; C, the turn-over and processing of truncated CFTRs; T70, T82, and T98 CFTR was assessed in a whole cell extract of a stably transfected BHK-21 cell using the indicated anti-CFTR mAb (L12B4, M3A7, or 24-1) and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). BHk lane, untransfected BHK-21 cell lysate; Q1 lane, lysate from CHO-BQ1 cells with stable, low-level expression of WT CFTR (26). B, the expression level of WT and mutant CFTRs was assessed in COS-1 cells 48 h post-transfection. All transfections were done with LipofectAMINE reagent. Detection was performed with mAbs Li2B4 and M3A7 and enhanced chemiluminescence. Note the different amounts of protein loaded as indicated. The COS lane contains untransfected COS-1 cell lysate. C, the turn-over and processing of core-glycosylated WT, T26, T70, T82, and T98 CFTR was examined in transiently transfected COS-1 cells by pulse-chase labeling. Following a 20-min pulse and a chase for the indicated time, CFTR was immunoprecipitated and visualized by fluorography. Filled arrowheads, completely glycosylated CFTR; open arrowheads, core-glycosylated CFTR. The doublet of the core-glycosylated CFTR is presumably caused by alternative initiation (14).

RESULTS AND DISCUSSION

Analysis of mutations found in the Cystic Fibrosis Genetic Consortium Database revealed that the shortest truncation, which manifests in CF with pancreatic insufficiency and recurrent pulmonary infection, is Q1412X (the genotype and clinical symptoms of the patient were kindly provided by C. J. Taylor, University of Sheffield, UK). Similar severe CF phenotype was also reported for frameshift mutations 4326delTC, 4279insA, and 4282insC (10). WT and truncated CFTRs bearing an N-terminal influenza HA epitope were expressed transiently in COS-1 cells and visualized with immunofluorescence using monoclonal anti-HA antibody (Babco, mAb 9E10) and fluorescein-conjugated donkey anti-mouse antibody. In contrast to F508 CFTR, open arrowheads, core-glycosylated CFTR; C, the turn-over and processing of truncated CFTRs (T26, T70, T82, and T98 CFTR) were examined in heterologous expression systems.

The subcellular distribution of T70, T82, and T98 CFTRs was examined first, because intracellular retention of mutant CFTRs by the ER quality control machinery is the most prevalent cause of CF (10). WT and truncated CFTRs bearing an N-terminal influenza HA epitope were expressed transiently in COS-1 cells and visualized with immunofluorescence using monoclonal anti-HA antibody. In contrast to ΔF508 CFTR, which displayed an intracellular, ER-like distribution pattern (Fig. 1, inset), both WT and truncated CFTRs were detectable at the cell surface and inside the cell (Fig. 1). Similar results were obtained for T70 CFTR stably expressed in BHK-21 cells (data not shown). These results suggest that intracellular retention is unlikely to be responsible for the impaired functional expression of truncated CFTRs.

To confirm that truncated CFTRs are functional at the plasma membrane, electrophysiological recordings were performed in the whole cell configuration on BHK-21 cells stably transfected with WT and mutant CFTRs. Expression of the mutants conferred cAMP-stimulated whole cell currents on BHK-21 cells (Fig. 2A). The linear current-voltage relationship under symmetrical conditions and the voltage-independent cAMP-stimulated current of the truncation constructs resembled the characteristics of WT CFTR (Fig. 2, A–C). Under symmetrical conditions, the reversal potentials (Vrev) of PKA-stimulated whole cell current of WT, T26, T70, T82, and T98 CFTR were –6.0 ± 3.6, –4.7 ± 2.8, –5.8 ± 2.7, –3.6 ± 1.8, and –3.8 ± 1.9 mV (mean ± S.E., n = 4–5), respectively. Upon imposing a 4-fold chloride concentration gradient directed from the extracellular compartment toward the cytosol, the Vrev for the same variants were as follows: –36.9 ± 0.5, –36.2 ± 0.5, –35.7 ± 0.35, –37.1 ± 0.4, and –36.1 ± 0.4, respectively. Considering that the predicted Vrev under symmetrical and asymmetrical conditions is 0 and –36.0 mV, respectively, these results suggest that the chloride ion selectivity of the truncated CFTRs is preserved. On the other hand, the mean cAMP-activated current density (picosiemperes/picofarad) for T70, T82, and T98 CFTR were only ~10% of the densities observed for WT CFTR in the presence of forskolin, IBMX, and cAMP-CAMP (Fig. 2D). Because similar current densities were obtained on two to three independent clones, we are confident that the ~90% reduction in current densities is a consequence of the truncation of CFTR rather than clonal variations. Importantly, the cAMP-activated current density of T26 was almost identical to that of WT CFTR, consistent with recent observations, indicating that deletion of the last 26 amino acid residues has no effect on the CFTR channel function (21).

<ref>Fig. 3.</ref>
TABLE I
Maturation efficiency and turn-over rate of truncated and WT CFTR

The maturation efficiency was assessed in experiments described in Fig. 3C using quantitative phosphor-image analysis and was expressed as the percent of core-glycosylated CFTR converted to complex-glycosylated CFTR, as described previously (18). Data are the means of a minimum of three to four independent experiments. The halflives of the core- and complex-glycosylated WT CFTR and truncated CFTRs were calculated from experiments performed with COS-1 and BHK-21 cells as shown in Figs. 3C and 4.

| CFTR | Maturation efficiency | 1/2 core-glycosylated | 1/2 complex-glycosylated |
|------|-----------------------|-----------------------|-------------------------|
|      | % ± S.E.               | h                     | h                       |
| WT   | 28 ± 2                | 0.7                   | 14                      |
| T26  | 28 ± 2                | 0.8                   | 12                      |
| T70  | 28 ± 2                | 0.8                   | 3.5                     |
| T82  | 22 ± 2                | 0.8                   | 3.5                     |
| T98  | 9 ± 1                 | 0.4                   | 3.6                     |

Fig. 4. Premature termination accelerates the degradation of the complex-glycosylated CFTR. A, the turnover of mature, complex-glycosylated WT and truncated CFTR was determined in BHK-21 stable transfectants. To assure the conversion of the core-glycosylated form into the complex-glycosylated CFTR, the initial sample (0 h) was taken 2 h after pulse labeling. The chase for WT CFTR and T26 was 24 h, whereas for T70, T82, and T98 CFTR it was 4 h. Determination of the half-lives of the complex-glycosylated WT and truncated CFTRs in stable (BHK, B) and in transient (COS-1, C) expression systems. The radioactivity remaining in the complex-glycosylated CFTR was measured by PhosphorImager analysis and is expressed as a percent of initial value. Data are the means ± S.E. of three or four independent experiments. Two or three clonally selected BHK-21 cell lines were included for each truncations.

To address the possibility that a decrease in the expression level of the truncated CFTRs accounts for the reduced current densities, the steady-state level of mutant CFTRs was determined with immunoblotting. Three monoclonal anti-CFTR antibodies (mAbs), L12B4, M3A7 (16), and 24-1 (17) were used, which recognize NBD1, NBD2, and the C-terminal five amino acid residues, respectively. Both core-glycosylated (band B, molecular mass ∼ 150 kDa) and complex-glycosylated (band C, molecular mass ∼ 170 kDa) WT and mutant CFTRs could be identified on the immunoblots of stably transfect BHK-21 cell extracts probed with mAbs L12B4, M3A7, and 24-1 (Fig. 3A). Successive truncations resulted in predictable alterations in the properties of CFTR; gradual decreases in the molecular mass of the core- and complex-glycosylated CFTRs, the inability of mAb 24-1 to recognize any of the truncated species, and the inability of mAb M3A7 to recognize T98 CFTR. According to densitometric analysis of immunoblots, the expression level of the complex-glycosylated T70, T82, and T98 CFTR decreased by 90–95% compared with WT CFTR in individual clones (Fig. 3B) as well as in the mixture of clones (not shown). The loss of CFTR expression from the cell surface manifested in the ∼ 90% reduction of the cAMP-activated chloride current densities (Fig. 2D). Comparable reduction was observed in the expression of T70, T82, and T98 CFTR in the COS-1 transient expression system detected with L12B4 and M3A7 antibodies (Fig. 3B), verifying the results obtained in stable transfectants.

Keeping in line with the whole cell current recordings and the data of Mickle et al. (21), the deletion of the last 26 residues had no impact on CFTR expression in transient or in stable transfectants (Figs. 2D and 3A, A and B). Although in the absence of single-channel recordings we cannot rule out the possibility that truncations alter the open probability (p_o) and/or unitary conductance of the CFTR, this seems unlikely because an ∼ 90% reduction in the expression level of WT CFTR has been associated with an ∼ 90% loss of the cAMP-stimulated whole cell current density in CHO-BQ1 cells (Figs. 2D and 3A).^4^ In principle, decreased stability of the mutant transcripts, defective post-translational folding, and accelerated degradation, or a combination of these processes, could explain the abrupt reduction in the expression levels of complex-glycosylated, mutant CFTRs. Thus, further experiments were done to determine which of these factors play a role. The rate of biosynthesis of the truncated CFTRs was determined by pulse labeling of the newly synthesized proteins and quantifying incorporation of label by immunoprecipitation and phosphor-image analysis. The results showed that the biosynthesis of the

^4^ Lowering the copy number and the expression of WT CFTR was achieved using 10 μM methotrexate during the clonal selection of CHO-BQ1 cells. To assure a high copy number of WT and mutant CFTR, BHK-21 cells were selected in medium supplemented with 500 μM methotrexate.
mutant CFTRs was not reduced compared with the WT rate (data not shown).

The folding efficiency of truncated CFTR was determined by monitoring the conversion of core-glycosylated protein to the complex-glycosylated form (Fig. 3A) (18). The processing efficiencies of transiently expressed T26, T70, and T82 CFTR were found to be between 22 and 28%, comparable with that of WT CFTR both in transient (Table I) and stable transfectants (17, 22). In contrast, the folding efficiency of T98 CFTR was decreased from 22 to 12%, indicating a 2-fold increase in the degradation rate for core-glycosylated T98 (Table I). This decrease is conceivable because T98 is the only truncation encroaching upon NBD2, the predicted second nucleotide binding domain, deleting a few of its C-terminal residues. Whereas the half-lives ($t_{1/2}$) of core-glycosylated T82, T70, and T26 and WT CFTR are $\sim$40 min, T98 has a $t_{1/2} \sim 20$ min (Table I). These data indicate that the C-terminal tail, encompassing the last 82 amino acid residues, are not essential for the post-translational folding and biosynthetic processing. Based on the increased steady-state level, which coincides with normal biosynthetic efficiencies of transiently expressed T26, T70, and T82 CFTR, we propose that the C-terminal tail is essential to maintain the stability of mature, complex-glycosylated CFTR.

To test this hypothesis, the turn-over of complex-glycosylated, truncated CFTR was assessed by pulse-chase labeling. As we anticipated, the $t_{1/2}$ of stably expressed complex-glycosylated T70, T82, and T98 CFTR was reduced 5- to 6-fold ($t_{1/2} \sim 1.6–2$ h), compared with T26 and WT CFTR ($t_{1/2} \sim 10–11$ h), as shown in Fig. 4, A and B. A comparable reduction in the $t_{1/2}$ of the complex-glycosylated form was observed in COS-1 cells transiently expressing the truncated CFTRs (Fig. 4C and Table I). The accelerated turn-over of the complex-glycosylated T70, T82, and T98 CFTRs, both in transient and stable expression systems, provides a plausible explanation for the reduction in their steady-state level and for the corresponding diminution in the cAMP-stimulated whole cell current density (Fig. 2D). These observations can account, at least in part, for the severe CF phenotype seen in compound heterozygote CF patients who have a $\Delta F508$ CFTR allele and an allele with a truncation mutation.

The classes of CF-associated mutations can be grouped into two major categories (4, 8). The first group includes those mutants that are unable to accumulate at the cell surface, either because of impaired biosynthesis (Class I and Class V), or because of defective folding at the ER (Class II). Mutants that belong to the second category are expressed at the cell surface but fail to translocate chloride ions because of a defect in activation (Class IV) or channel conductance (Class III). Because the biosynthetic processing and macroscopic chloride channel function of some of the truncated CFTR constructs appear to be normal but the biological stability of their mature, complex-glycosylated form is dramatically reduced, we propose a third category of mutations (putatively designated Class VI), which would include stability mutants such as those characterized in the present work.

The molecular mechanism underlying the stabilization of CFTR by the C-terminal tail remains to be resolved. The C-terminal tail of CFTR might confer stability on CFTR by several mechanisms or a combination thereof. For example, C-terminal truncation could facilitate lysosomal degradation of the mutant CFTR by exposing endo-lysosomal sorting motifs (23, 24), or it might prevent efficient recycling from the endosomal compartment back to the cell surface (25, 26). The absence of the C-terminal tail may structurally destabilize folded CFTR, increasing the portion of non-native molecules that are susceptible to proteolysis (27, 28). Recent evidence suggests that the last five residues at the C terminus can bind to NHERF or to its human homologue, EBFP50 (29–31). It was speculated that the association of CFTR with NHERF may stabilize CFTR by tethering it to the cytoskeleton. However, the physiological significance of this interaction has to be further investigated in the light of the normal lung and pancreatic function of patients homozygous for the deletion of the C-terminal 26 residues (21). Nevertheless, our results are the first to highlight the role of the C-terminal domain in determining the expression level of CFTR by increasing the metabolic stability of CFTR in post-ER compartments and to provide an explanation for the severe phenotype of CF patients harboring premature truncations. In the broader context of pathomechanisms of genetic disease, 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 biogenesis but also by accelerating the degradation of a fully processed, functional protein.

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