Biogenesis, Maturation and Surface Trafficking of Wild-Type and Mutant CFTR

Ph.D. Dissertation

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INTRODUCTION

Cystic fibrosis (CF) is the most prevalent hereditary disease among Caucasians. It is an autosomal recessive genetic disorder resulting in a fatal outcome in most cases. The affected sites are mainly the exocrine glands with a consequent disfunction of the lungs, the pancreas, the GI tract and the reproductive system. Approximately 1:25 people of European, 1:22 of Ashkenazi Jewish, 1:46 of Hispanic 1:65 of African and 1:90 of Asian descent carry at least one mutated CFTR allele, and about 30,000 people in the United States are living with CF.

CF caused by mutations in the 180-kb cystic fibrosis transmembrane conductance regulator (CFTR) gene located on the long (q) arm of chromosome 7 at position 31.2. The gene product, the CFTR protein is a 168 kDa multidomain chloride channel that belongs to the adenosine triphosphate (ATP)-binding casette (ABC) transporter super-family. It is expressed in a wide variety of cell types, but it is most abundant at the apical surface of secretory epithelia, where it functions as part of a large macromolecular protein complex. CFTR, also referred to as ABC transporter ABCC7, plays a significant role in electrolyte and fluid movement regulation across epithelial cell layers. Although CFTR is not the only chloride channel in these tissues, its current is critical in maintaining transepithelial osmotic balance. Functional insufficiency of the mutated CFTR protein gives rise to CF symptoms such as pancreatic insufficiency, high salt concentration in sweat, thick, dehydrated mucus in the airways with frequent upper respiratory tract infections and consequential respiratory failure. Obstruction or absence of the vas deferens in male, and reduced fertility in female CF patients are well known complications of the disease as well.CF is a monogenetic disorder, and its clinical severity varies widely. There are more than 1,500 mutations listed in the CFTR database (http://www.genet.sickkids.on.ca/cftr), but a 3 bp deletion resulting in a loss of a phenylalanine residue at position 508 (ΔF508) is the most prevalent disease causing mutation and is responsible for more than 70% of all CF cases.

CFTR is comprised of two homologous halves, with each half containing a large membrane-spanning region with six transmembrane segments (TM1 and TM2) and a

nucleotide –binding domain (NBD1 and NBD2). The two halves are separated by a large regulatory domain (R) containing multiple consesnsus phosphorilation sites (Bradbury et al., 1992). A schematic diagram of the CFTR structure is shown in Figure 1A. Two asparagine residues of the CFTR protein are N-glycosylated in the endoplasmic reticulum, and after proper folding, the protein traffics to the Golgi apparatus from the ER and the carbohydrate chains are modified in the trans-Golgi network to their mature form (O'Riordan et al., 2000). Mature, fuly glycosylated CFTR leaves the Golgi and directly travels to the apical cell membrane or to the recycling endosomes (Bertrand and Frizzell, 2003). Within any pool of CFTR expressed in cells there is a mixture of core glycosylated (ER form) and complex glycosylated (post-Golgi form) CFTR. The differentially glycosylated forms can be distinguished by the difference of their molecular weights subjected to denaturing SDS-PAGE electrophoresis. Figure 1B. showes the two CFTR forms as the ER form or Band B, and post-Golgi form or Band C.

AIMS AND HYPOTHESES

To test the hypotheses that the isoleucine residue at position 1427 is important for CFTR endocytosis and that ablation of this putative internalization signal Y*XX*I would increase the steady-state surface expression of CFTR. To test if these substitutions have any effect on the chloride channel properties of CFTR

To monitor the maturation efficiency of CFTR in heterologous expression systems such as HeLa and COS-7 cells and in two human epithelial cell lines that endogenously expresses CFTR, Calu-3 (Shen et al., 1994), and T84 cells (Cohn et al., 1992) using metabolic pulse-chase analysis of CFTR in these cells grown under both nonpolarizing and polarizing conditions, and to compare quantitative cell surface levels. Based on recent results that epithelial specific factors regulate both CFTR biogenesis and function, we hypothesized that CFTR biogenesis in endogenous CFTR expressing epithelial cells may be more efficient.

To determine whether the surface defects exhibited by rΔF508 CFTR are also TS, and therefore possibly related to the ER folding defect. Additionally, to study if treatment of rΔF508 CFTR with chemical compounds known to promote rescue can affect ΔF508 CFTR

cell-surface properties, such as surface stability. Our hypothesis was that permissive temperature culture and small molecular correctors not only can rescue ΔF508 CFTR from ERAD, but also stabilizes it at the cell surface.

MATERIALS AND METHODS

COS-7 cells were cultured in modified Eagle's medium (Invitrogen) with 10% FBS at 37 °C in a humidified incubator in 5% CO_2 and transiently transfected using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's directions. The cells were incubated at 37 °C in a humidified incubator for 24–48 h before analysis.

HeLa cells overexpressing wild type CFTR were transduced and selected as previously described(Wu et al., 2000, Kappes et al., 2003), cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FBS at 37 °C in a humidified incubator in 5% CO_2. For cell monolayers, Calu-3 cells were seeded on 6.5- or 12-mm diameter Transwell filters (Corning-Costar, Corning, NY). After 2-3 days, the medium containing 10% FBS was exchanged to 2% FBS containing media, and cells were cultured for an additional 7-9 days with liquid both at the apical and the basolateral compartments. Under these conditions, the cells formed monolayers with trans-epithelial resistances of >800 $\Omega \cdot cm^2$.

HeLaΔF (where DF indicates a cell line expressing ΔF508 CFTR), *HeLaWT, CFBE41o$^-$ΔF and CFBE41o$^-$WT cell* lines were developed and cultured as described previously(Bebok et al., 2005). HeLa cells were grown in Eagle's modification of MEM (minimal essential medium; Invitrogen) supplemented with 10% (v/v) FBS (fetal bovine serum). Calu-3 cells were obtained from A.T.C.C. and were maintained in Eagle's modification of MEM supplemented with 10% (v/v) FBS, 2 mM glutamine, 1 mM pyruvate and 0.1 mM non-essential amino acids. CFBE41o$^-$ cell cultures were maintained in DMEM (Dulbecco's modified Eagle's medium) Ham's F12 medium (50:50, v/v) (Invitrogen) with 10% (v/v) FBS. For experiments requiring polarized cells, Calu-3 CFBE41o$^-$ΔF and CFBE41o$^-$WT cells were seeded on to 12 mm diameter Transwell filters (Costar, Corning). Under these

conditions, the cells formed polarized monolayers with transepithelial resistances of >1000 W/cm^2, as measured by a Millicell electrical resistance system (Millipore).

Small molecular correctors were provided by Cystic Fibrosis Foundation Therapeutics (Bethesda, MD, U.S.A.). Compounds tested were CFcor-325 (VRT-325, 4-cyclohexyloxy-2-{1[4-(4-methoxy-benzensulfonyl)-piperazin-1-yl]-ethyl}-quinazoline) (Loo et al., 2006, Van Goor et al., 2006)and Corr-4a ({2-(5-chloro-2-methoxy-phenylamino)-4´-methyl-[4,5´]-bithiazolyl-2´-yl}-phenyl-methanone) (Pedemonte et al., 2005). Both compounds were used at a 10 mM stock concentration in DMSO and a 10 mM working concentration in OPTIMEM medium (Invitrogen) supplemented with 2% (v/v) FBS. The presence of the vehicle (0.1% DMSO) in the medium did not mediate ER escape of ΔF508 CFTR in control samples, or facilitate changes in internalization. In all experiments, control samples contained the DMSO vehicle.

To label surface CFTR with Biotin the cells were cooled to 4^{o}C, washed with phosphate-buffered saline containing 1.0 mM MgCl2 and 0.1mM CaCl2 (PBS c/m), and incubated for 30 min with 10 mM NaIO4 in the dark. The cells were again washed with PBS c/m and labeled with 2 mM biotin-LC-hydrazide in 100 mM sodium acetate (pH 5.5) for 30 min. These labeled cells were extensively washed with PBS c/m and lysed in RIPA lysis buffer. After biotinylation and lysis, samples were divided into two equal samples and immunoprecipitated with anti-CFTR nucleotide binding domain 1 antibody and protein A-agarose. One of the immunoprecipitated samples was then eluted from the beads using Laemmli sample buffer (without bromphenol blue), diluted in RIPA buffer (150 mm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmTris-HCl, pH 8.0) 10-fold, and the biotinylated fraction was captured with avidin-Sepharose beads (Pierce) overnight at 4^{o}C. Both total CFTR and biotinylated CFTR were then *in vitro* phosphorylated using [γ^{32}P]ATP (PerkinElmer Life Sciences) and cAMP-dependent protein kinase (Promega).

RESULTS

Ablation of Internalization Signals in the C-terminal Tail of CFTR enhances Surface expression

The percentage CFTR at the cell surface was markedly increased for Y1424A,I1427A CFTR compared with both wild-type (108% increase, $n = 10$,$p < 0.001$) and Y1424A CFTR (59% increase, $n = 10$, $p < 0.001$) (Fig. 2,*bottom panel*). The surface biotinylation data indicated that modification of residues Tyr^{1424} and Ile^{1427}increased the steady-state surface expression of CFTR. The potential mechanisms that could account for these differences include changes in 1) maturation efficiency, 2) protein half-life, or 3) internalization and/or recycling rates.

Regarding the maturation efficiency we did not find differences, and the half-lives for wild-type (*Wt*), Y1424A, and Y1424A,I1427A CFTR were 10.3 ± 2.3, 11.3 ± 2.6, and 11.3 ± 1.5 h (mean ± S.D.). These findings indicated that the elevated surface expression of the mutants was not attributed to enhanced maturation or protein half-life.

For wild-type CFTR, 34% of the surface pool was internalized in 2.5 min. For Y1424A and Y1424A,I1427A CFTR, internalization dropped to 21 and 8%, respectively, during the same time period. These results demonstrate that CFTR endocytosis is inhibited by 76% when these two residues are modified.

The whole cell and single channel recordings together show that the difference in Cl^- channel activity is attributed to elevated surface expression without a significant change in CFTR chloride channel properties among wild-type, Y1424A, and Y1424A,I1427A CFTR.

Intracellular Processing of CFTR is Efficient in Epithelial cell Lines

In COS-7 cells, CFTR expression levels are dependent upon transfection efficiency, while in HeLa and Calu-3 cells, the expression levels were consistently high. The results of the real-time experiments establish that CFTR message levels in HeLa cells are 3.5-fold higher than

in Calu-3 cells. Our results indicate that although less CFTR was being produced in Calu-3 cells, the protein was processed much more efficiently to the mature form when compared to HeLa cells.

Results using peroteosomal inhibitoprs support our hypothesis that in Calu-3 cells all newly synthesized, core-glycosylated CFTR is processed to the fully glycosylated form, and therefore, there is no role for the proteasome in the early events of CFTR processing in Calu-3 cells.

Results using surface biotinylation assay demonstrate that the surface CFTR pool in Calu-3 cells is higher than in HeLa cells, but cell polarity does not affect the relative surface pool.

Metabolic pulse-chase experiments showed that efficient processing of endogenous wild type CFTR is not a unique feature of Calu-3 cells but also exists in a colonic epithelial cell line (T84).

Pharmacological Chaperones Enhance Surface Stability of ΔF508 CFTR

Our results demonstrate that a significant amount of fully glycosylated CFTR is formed during 27 °C culture. To confirm that the fully glycosylated rΔF508 CFTR was expressed at the cell surface, cells were surface biotinylated using biotin–LC–hydrazide. Biotinylated CFTR was detected in HeLaDF cells after 27 °C culture, but not in cells maintained at 37 °C.

We found that the half-life of WT CFTR is 12±1.5 h, whereas the half-life of rΔF508 CFTR is 4±1 h on restrictive (37°) temperature. The rΔF508 CFTR protein half-life is much shorter than that of WT CFTR, and these results are in agreement with previously published experiments performed in BHK (baby-hamster kidney) and CHO (Chinese-hamster ovary) cells.

We found that at 27 °C, the half-lives of WT CFTR and rΔF508 CFTR were 60±11 h and 63±9 h respectively (Fig. 12C), demonstrating that the instability of rΔF508 CFTR

compared with WT CFTR which was observed at the restrictive temperature is not apparent at the permissive temperature; in other words, the rΔF508 CFTR half-life defect is a TS defect.

In HeLa cells both WT CFTR and rΔF508 CFTR endocytosis rates are rapid, with 25±5% and 27±5% of the surface pool internalized in 2.5 min respectively (Fig. 13A). This result is consistent with a previous comparison of WT CFTR and rΔF508 CFTR internalization rates in BHK cells (Sharma et al., 2004).

In CFBE41o$^-$ cells we found that rΔF508 CFTR surface stability is decreased compared with WT CFTR, and polarization did not affect the surface stability of either protein.

Our results in epithelial cells indicated that 27 °C treatment eliminated the difference in internalization rates between WT CFTR and rΔF508 CFTR. Importantly, the results of these studies indicate that both the short surface half-life and the rapid internalization rate of rΔF508 CFTR are TS defects.

To study the effect of small molecular weight correctors internalization assays were performed at 37 °C. The results indicated that both CFcor-325 and Corr-4a decreased the internalization rate of rΔF508 CFTR from 30% to ~5% and ~1% respectively (P<0.005, Fig. 17, top and middle panels). Interestingly, the compounds had no effect on WT CFTR endocytosis or TR endocytosis from the apical, suggesting that the effect was specific for rΔF508 CFTR.

In surface stability experiments CFcor-325 extended the half-life of rΔF508 CFTR from 2.5±0.4 h to 4.6±0.9 h, and Corr-4a extended the half-life from 2.6±0.6 h to 4.5±1.2 h, indicating that both compounds stabilized the half-life of rΔF508 CFTR. Significantly, neither compound affected the half-lives of neither WT CFTR nor TR, suggesting the effects observed are specific for rΔF508 CFTR.

CONCLUSIONS

Here we show that a second substitution in the carboxyl-terminal tail of CFTR, I1427A, on Y1424A background more than doubles CFTR surface expression as monitored by surface biotinylation. Internalization assays indicate that enhanced surface expression of Y1424A, I1427A CFTR is caused by a 76% inhibition of endocytosis. Patch clamp recording of chloride channel activity revealed that there was a corresponding increase in chloride channel activity of Y1424A, I1427A CFTR, consistent with the elevated surface expression, and no change in CFTR channel properties. Y14124A showed an intermediate phenotype compared with the double mutation, both in terms of surface expression and chloride channel activity. Metabolic pulse-chase experiments demonstrated that the two mutations did not affect maturation efficiency or protein half-life. Taken together, our data show that there is an internalization signal in the COOH terminus of CFTR that consists of Tyr (1424)-X-X-Ile(1427) where both the tyrosine and the isoleucine are essential residues. This signal regulates CFTR surface expression but not CFTR biogenesis, degradation, or chloride channel function.

One unusual feature of this protein is that during biogenesis, approximately 75% of wild type CFTR is degraded by the endoplasmic reticulum (ER)-associated degradative (ERAD) pathway. Examining the biogenesis and structural instability of the molecule has been technically challenging due to the limited amount of CFTR expressed in epithelia. Consequently, investigators have employed heterologous overexpression systems. Based on recent results that epithelial specific factors regulate both CFTR biogenesis and function, we hypothesized that CFTR biogenesis in endogenous CFTR expressing epithelial cells may be more efficient. To test this, we compared CFTR biogenesis in two epithelial cell lines endogenously expressing CFTR (Calu-3 and T84) with two heterologous expression systems (COS-7 and HeLa). Consistent with previous reports, 20 and 35% of the newly synthesized CFTR were converted to maturely glycosylated CFTR in COS-7 and HeLa cells, respectively. In contrast, CFTR maturation was virtually 100% efficient in Calu-3 and T84 cells. Furthermore, inhibition of the proteasome had no effect on CFTR biogenesis in Calu-3 cells, whereas it stabilized the immature form of CFTR in HeLa cells. Quantitative reverse transcriptase-PCR indicated that CFTR message levels are approximately 4-fold lower in Calu-3 than HeLa cells, yet steady-state protein levels are comparable. Our results

question the structural instability model of wild type CFTR and indicate that epithelial cells endogenously expressing CFTR efficiently process this protein to post-Golgi compartments.

Misfolded proteins destined for the cell surface are recognized and degraded by the ERAD [ER (endoplasmic reticulum) associated degradation] pathway. TS (temperature-sensitive) mutants at the permissive temperature escape ERAD and reach the cell surface. In this present paper, we examined a TS mutant of the CFTR [CF (cystic fibrosis) transmembrane conductance regulator], CFTR ΔF508, and analysed its cell-surface trafficking after rescue [rΔF508 (rescued ΔF508) CFTR]. We show that rΔF508 CFTR endocytosis is 6-fold more rapid (~30% per 2.5 min) than WT (wild-type, ~5% per 2.5 min) CFTR at 37 °C in polarized airway epithelial cells (CFBE41o$^-$). We also investigated rΔF508 CFTR endocytosis under two further conditions: in culture at the permissive temperature (27 °C) and following treatment with pharmacological chaperones. At low temperature, rΔF508 CFTR endocytosis slowed to WT rates (20% per 10 min), indicating that the cell-surface trafficking defect of rΔF508 CFTR is TS. Furthermore, rΔF508 CFTR is stabilized at the lower temperature; its half-life increases from <2 h at 37 °C to >8 h at 27 °C. Pharmacological chaperone treatment at 37 °C corrected the rΔF508 CFTR internalization defect, slowing endocytosis from ~30% per 2.5 min to ~5% per 2.5 min, and doubled ΔF508 surface half-life from 2 to 4 h. These effects are ΔF508 CFTR-specific, as pharmacological chaperones did not affect WT CFTR or transferrin receptor internalization rates. The results indicate that small molecular correctors may reproduce the effect of incubation at the permissive temperature, not only by rescuing ΔF508 CFTR from ERAD, but also by enhancing its cell-surface stability.

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