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Targeted Proteomic Quantitation of the Absolute Expression and Turnover of Cystic Fibrosis Transmembrane Conductance Regulator in the Apical Plasma Membrane

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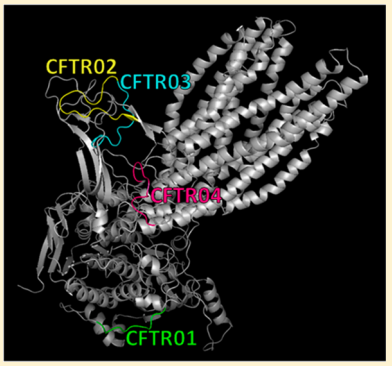
S *Supporting Information*

ABSTRACT: Deficient chloride transport through cystic fibrosis (CF) transmembrane conductance regulator (CFTR) causes lethal complications in CF patients. CF is the most common autosomal recessive genetic disease, which is caused by mutations in the CFTR gene; thus, CFTR mutants can serve as primary targets for drugs to modulate and rescue the ion channel's function. The first step of drug modulation is to increase the expression of CFTR in the apical plasma membrane (PM); thus, accurate measurement of CFTR in the PM is desired. This work reports a tandem enrichment strategy to prepare PM CFTR and uses a stable isotope labeled CFTR sample as the quantitation reference to measure the absolute amount of apical PM expression of CFTR in CFBE 41o- cells. It was found that CFBE 41o- cells expressing wild-type CFTR (wtCFTR), when cultured on plates, had 2.9 ng of the protein in the apical PM per million cells; this represented 10% of the total CFTR found in the cells. When these cells were polarized on filters, the apical PM expression of CFTR increased to 14%. Turnover of CFTR in the apical PM of baby hamster kidney cells overexpressing wtCFTR (BHK-wtCFTR) was also quantified by targeted proteomics based on multiple reaction monitoring mass spectrometry; wtCFTR had a half-life of 29.0 ± 2.5 h in the apical PM. This represents the first direct measurement of CFTR turnover using stable isotopes. The absolute quantitation and turnover measurements of CFTR in the apical PM can significantly facilitate understanding the disease mechanism of CF and thus the development of new disease-modifying drugs. Absolute CFTR quantitation allows for direct result comparisons among analyses, analysts, and laboratories and will greatly amplify the overall outcome of CF research and therapy.



KEYWORDS: *Cystic fibrosis, cystic fibrosis transmembrane conductance regulator, membrane protein quantitation, quantitative proteomics*

1. INTRODUCTION

For cystic fibrosis (CF), the most common autosomal recessive genetic disease, there is a recent paradigm shift from treating disease symptoms toward developing drugs that repair fundamental defects in the anion channel of cystic fibrosis transmembrane conductance regulator (CFTR).[1−3] CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily and functions as a cAMP-dependent, protein kinase-activated Cl^- channel in the plasma membrane (PM). It has five structural domains: two transmembrane domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2), and a unique regulatory domain (RD). Deletion of a phenylalanine at position 508 (F508del) is the most frequent mutation, accounting for ∼90% of the CF population. The F508del mutation is located in NBD1, putatively interfacing with TMDs, and impairs coupled domain folding, PM expression, ion channel function, and protein stability.[4,5] The misfolded mutant is retained inside cells at the endoplasmic reticulum (ER), and is degraded by the proteasome.[6] Evidence suggests that, during biosynthesis and trafficking of CFTR, different domains of the protein interact with various chaperone systems, facilitating the maturation of wild-type

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CFTR (wtCFTR) and the recognition of the F508del mutant. Thus, F508del expression in the apical PM is minimal; consequently, F508del CF patients do not have sufficient CFTR channel activity in epithelial cells in their airways, intestine, pancreas, sweat ducts, testes, and other fluid-transporting tissues.[7,8]

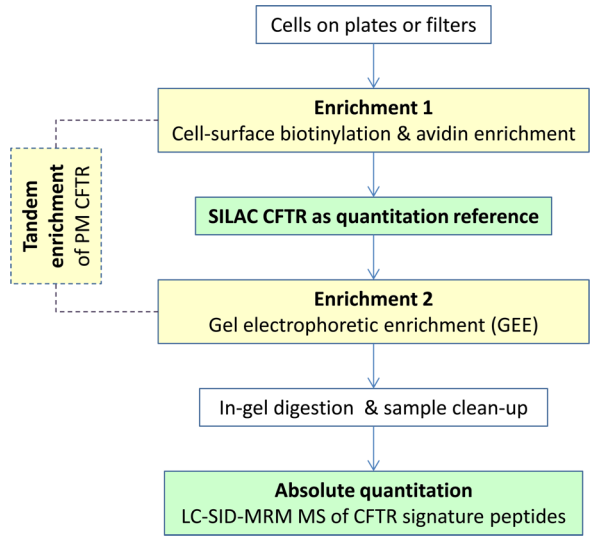
Two major categories of new CFTR modulator drugs are currently under extensive research and clinical investigations.[9] Corrector drugs repair the biogenesis, trafficking, and ultimately the apical PM expression of the protein, and potentiator drugs enable the channel function of mutant CFTR proteins in the PM. Corrector drugs like VX-809 (Lumacaftor) repair folding and trafficking of CFTR mutants and enhance the apical PM expression of F508del.[2,10,11] The rescue of PM expression of mutant CFTR, like F508del, by corrector drugs is the first step toward restoring Cl^- channel activity. However, VX-809 has limited clinical benefit for F508del CF patients, and its mechanism of action has yet to be fully understood.[2,12] In addition, it is generally agreed that the first generation of CF drugs has reached an apparent therapeutic ceiling.[2,3,13−16] In-depth analysis of how current investigational drugs work will provide mechanistic insights for developing future CF medicines. These studies would be greatly enhanced by accurate measurements of CFTR and its mutants in cells, especially in the PM, where the protein performs its vital ion channel function.

Difficulties in the analysis of CFTR are among the major challenges for molecular investigations of CF drug action. CFTR is a large, glycosylated, multidomain, and low-abundance integral membrane protein. In order to evaluate the efficacy of drug compounds, efficient methods are needed to detect and quantify changes in PM CFTR expression. Historically, western blot analysis of CFTR has been the central CFTR quantitation method in CF research;[17] newly developed antibodies have greatly improved the sensitivity of this method.[18] However, the reproducibility, precision, accuracy, and robustness of the method are less than ideal, providing only semiquantitative measurements, with a relatively large coefficient of variance and a narrow dynamic range. Furthermore, CFTR aggregates and degrades during sample preparations, leading to large measurement variations. These critical problems with membrane proteins present major, intrinsic challenges for analysis.[19−21] Other methods for quantifying surface expression of CFTR require engineered cells [e.g., green fluorescent fusion proteins (GFP)] and are not directly applicable to primary cells, which are better systems for testing new experimental drugs.

Technology innovations, from genomics[22] to proteomics,[23,24] have played important roles in advancing CF research and therapy. We have developed the first quantitative method to mitigate the intrinsic analytical problems with full-length CFTR and use CFTR signature peptides as measurement surrogates.[25] CFTR is digested into peptides that are relatively easy to prepare for mass spectrometry (MS) quantitation. CFTR quantified in this work ranges from a few tens of picograms to low nanograms per million baby hamster kidney cells overexpressing wtCFTR (BHK-wtCFTR) or human colorectal adenocarcinoma cells (HT-29).[25] A signature peptide is selected for quantitation by a method of liquid chromatography−stable isotope dilution−multiple reaction monitoring MS (LC−SID−MRM MS). It is the method of choice for quantifying target proteins in complex biomatrices,[26−28] which is regarded as the MS version of western blot analysis, and has comparable sensitivity to that of western blot analysis but superior specificity. MRM MS monitors gas-phase dissociation reactions of target analytes, which requires sequential detection of an analyte precursor ion as the reactant followed by one or several analyte fragment ions as the products; therefore, it is highly specific and can be applied to complex samples with minimal component separation. The sensitivity and specificity afforded by the MRM MS method removes the dependence on high-quality antibodies for conventional immunoassays of protein targets like CFTR.[18]

However, later addition of peptide quantitation reference standards cannot be used to normalize (1) differential sample loss during CFTR enrichment (CFTR has low concentration in the PM, and it is essential to enrich the protein to obtain the needed quantitation limit) and (2) variations in proteolytic digestion of CFTR (membrane proteins are difficult to digest completely).[29] Thus, peptide-level quantitation reference standards are less than ideal for the absolute quantitation of CFTR. More reliable absolute quantitation of PM CFTR will enable accurate and precise sample-to-sample, day-to-day, analyst-to-analyst, and lab-to-lab comparisons of results, similar to that shown for LC−SID−MRM MS quantitation of plasma proteins,[26] and significantly facilitate the development of new CF drugs. Herein, we report a general workflow (Scheme 1) for the enrichment and absolute quantitation of large, low-abundance CFTR protein (and other membrane proteins) in the apical PM (broadly referred to as the fraction of PM that is not in contact with the culture support and thus is accessible to cell surface biotinylation) of BHK-wtCFTR and CFBE cells. With a variation of this method, CFTR turnover in the apical PM of BHK-wtCFTR cells is measured for the first time without using radioisotopes.

Scheme 1. Tandem Enrichment of CFTR in the PM and Workflow for Targeted Proteomic Quantitation of Its Absolute Expression



2. EXPERIMENTAL SECTION

2.1. Materials

Urea, Tris base, iodoacetamide, acrylamide/bis-acrylamide solution (40%, 29:1), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, L-leucine, L-lysine, L-arginine, methotrexate, doxycycline, protease inhibitor cocktail, sodium orthovanadate, 1 M Tris-

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HCl buffer solution, phosphate buffer saline (PBS, 10×, pH 7.4), Triton X-100, and the anhydrides were purchased from Sigma-Aldrich (St. Louis, MO). Pierce cell surface protein isolation kit, dithioerythritol (DTE), β-mercaptoethanol (β-ME), trifluoroacetic acid (TFA), ammonium bicarbonate, ammonium persulfate, formic acid, and high-performance liquid chromatography (HPLC) grade acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). The peptide CFTR01 was synthesized by AnaSpec (San Jose, CA) or Peptide 2.0 (Chantilly, VA). Isotopic CFTR01 peptide (NSILTET[L-$^{13}C_6{}^{15}N$]HR was synthesized by Cambridge Research Biochemicals (Cleveland, UK). ^{18}O-Water (>97%) was purchased from Cambridge Isotope Laboratories (Andover, MA) or given as a gift from Olinax (Hamilton, ON, Canada). Water was obtained from a Milli-Q ultrapure water purification system (Millipore, Billerica, MA). Proteomics grade recombinant trypsin was purchased from Roche Applied Science (Indianapolis, IN). Human embryonic kidney cell line (HEK293F) stably transfected with fused cDNA for wtCFTR and GFP (HEK293F-D042-JK), HEK293F-wtCFTR, was a gift from Dr. John C. Kappes (University of Alabama at Birmingham). A cystic fibrosis bronchial-derived cell line complimented with a 4.7 kb wild-type CFTR cDNA (CFBE 41o-/pCEP-CFTR N 4.7 kb) and a cystic fibrosis bronchial-derived cell line complimented with a 4.7 kb F508del CFTR cDNA (CFBE 41o-/pCEP-CFTR F508del 4.7 kb) were from Dr. Dieter Gruenert (University of California at San Francisco).[30] The HT-29 cell line was purchased from American Type Culture Collection (Manassas, VA). Purified full-length CFTR samples were gifts from Dr. L. J. DeLucas (University of Alabama at Birmingham) and Dr. J. He (Accelagen, San Diego, CA). Purification of the later full-length CFTR sample was performed according to a procedure developed by Dr. J. R. Riordan at the University of North Carolina, Chapel Hill. The absolute concentration of this highly purified CFTR sample was obtained by amino acid analysis, and this sample was used as the reference standard to quantify stable isotope labeled CFTR samples prepared in-house (see the following section). Amino acid analysis for the highly purified, native CFTR was performed at the Keck Biotechnology Resource Laboratory at Yale University. DMEM, Ham's F-12, DME/Low media deficient in L-arginine, L-leucine, and L-lysine, and fetal bovine serum were purchased from Thermo Scientific HyClone (Logan, UT). Dialyzed fetal bovine serum was purchased from Life Technologies (Thermo, Carlsbad, CA).

2.2. Cell Culture and Preparation of Protein Quantitation Standard

BHK-wtCFTR cells were cultured in DMEM/Ham's F-12 with 550 μM methotrexate and 10% of each of the following reagents: fetal bovine serum, pen−strep, nonessential amino acids, and sodium pyruvate. The heavy isotope labeled, full-length CFTR was obtained by the method of stable isotope labeling by amino acids in cell culture (SILAC)[31] from BHK-wtCFTR cells cultured in DME/Low deficient in L-lysine, L-leucine, and L-arginine supplemented with 550 μM methotrexate, 0.4 mM L-arginine·HCl, 0.8 mM L-lysine·HCl, 0.8 mM L-leucine-1,2-^{13}C (Cambridge Isotopes, Andover, MA), and 10% dialyzed fetal bovine serum. Cells grown in this labeled media were allowed at least 7−8 doublings prior to harvesting. CFBE 41o-/pCEP-CFTR N 4.7 kb cells were grown in MEM medium with 0.3 g/L L-glutamine, 1 g/L glucose, 2.2 g/L $NaHCO_3$, 10% fetal bovine serum, 20 mM sodium pyruvate, 10 000 μg/mL streptomycin, and 10 000 units/mL penicillin. The CFBE cells were grown in plates or snapwell filters (Sigma-Aldrich, St. Louis, MO) that were coated with human fibronectin (Fisher Scientific, Fair Lawn, NJ). The HEK293F-wtCFTR cells stably transfected with wtCFTR-GFP fused cDNA were cultured using DMEM with 10% fetal bovine serum, and doxycycline was added at 0, 0.25, 0.75, or 1.00 μg/mL the night before cell harvesting.

2.3. Cell Surface Biotinylation and Cell Lysate Fractionation

Surface biotinylation[25,32] was performed on cells using the surface protein isolation kit from Pierce, following the manufacturer's instructions. Briefly, cells were first labeled with a thiol-cleavable, amine-reactive biotinylation reagent [sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin)] and quenched. The biotinylated cells were dislodged by trypsinization or scraping from the plates or filters and subsequently lysed with buffer. The lysis buffer (800 μL) containing 1% (v/v) Triton X-100, 150 mM NaCl, 20 mM Tris-HCl at pH 7.2, and 1% (v/v) protease inhibitors was used for the lysis of 12−14 million BHK-wtCFTR cells or 6−8 million CFBE cells. The labeled proteins were captured on avidin agarose and released using Triton X-100 buffer containing 50 mM dithiothreitol (DTT).

2.4. Gel-Based Electrophoretic Enrichment (GEE)

Gel electrophoresis was performed using a resolving gel consisting of 8% acrylamide and 0.2% bis(acrylamide). BioRad glass plates with 1.0 mm spacer plates and a 10-well comb were used for analysis of overexpressed CFTR; a single well allowing for a 20 μL loading volume was sufficient for analysis of overexpressed CFTR in about 8 μg of total biotinylated protein. For endogenous CFTR, 1.5 mm spacer plates and a 2-well comb consisting of 1 preparative well for sample loading (680 μL, 500 μg of total biotinylated proteins) and 1 well for loading the standard molecular weight protein marker or purified CFTR were used. For the turnover study, 1.5 mm spacer plates and a 5-well (120 μL) comb were used. CFTR samples were mixed with aemmli sample buffer containing 20% (v/v) β-ME and incubated at 30 °C for 30 min. Gel electrophoresis was carried out at 200 V constant voltage for 45 min or until the dye front reached the bottom of the gel.

2.5. Digestion

Gel pieces at the interface between the resolving and stacking gels (~1 mm^3) were excised for further sample preparation. Gel pieces were washed (25 mM ammonium bicarbonate in H_2O/acetonitrile), reduced (10 mM DTE), and alkylated (20 mM iodoacetamide) before trypsinization (20 ng/μL). The resulting digestion solution was dried via SpeedVac (Savant, Farmingdale, NY) and overnight lyophilization (Labconco, Kansas City, MO).

2.6. LC−SID−MRM MS

The optimization and data analysis of this work were assisted by the Skyline software.[33] The preparation of stable isotope reference ^{18}O(Δ4)-CFTR01 and LC−SID−MRM MS quantitation were performed following previously reported procedures.[25] Some exceptions were those experiments where CFTR protein quantitation was accomplished in CFBE and BHK-wtCFTR cells. In those cases, an Eksigent NanoLC-Ultra 2D$^+$ (Redwood City, CA) was used at a flow rate of 400 nL/min with a self-pack Picofrit column (New Objective, Woburn, MA) filled with 2.7 μM diameter, 160 Å pore Halo resin (MacMod,

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Table 1. CFTR Signature Peptides and Isotopic Counterparts with Respective Transitions for LC−SID−MRM MS Analysis[a]

peptide	origin	sequence	transition type
CFTR01	Native	NSILTETLHR	$[M + 2H]^{2+} \rightarrow y_7, y_6, y_5$
L(Δ4)-CFTR01	SILAC	NSI[L-1,2-$^{13}C_2$]TET[L-1,2-$^{13}C_2$]HR	
R(Δ6)-CFTR01	SILAC	NSILTETLH[R-$^{13}C_6$]	
^{18}O(Δ4)-CFTR01	Synthetic, ^{18}O-Labeling	NSILT[E-$^{18}O_2$]TLH[R-$^{18}O_2$]	
L(Δ7)-CFTR01	Synthetic	NSILTET[L-$^{13}C_6{}^{15}$N]HR	
CFTR02	Native	LSLVPDSEQGEAILPR	$[M + 2H]^{2+} \rightarrow y_{12}, y_{10}, y_9$
L(Δ6)-CFTR02	SILAC	[L-1,2-$^{13}C_2$]S[L-1,2-$^{13}C_2$]VPDSEQGEAI[L-1,2-$^{13}C_2$]PR	
R(Δ6)-CFTR02	SILAC	LSLVPDSEQGEAILP[R-$^{13}C_6$]	
CFTR03	Native	ISVISTGPTLQAR	$[M + 2H]^{2+} \rightarrow y_8, y_7, y_6$
R(Δ6)-CFTR03	SILAC	ISVISTGPTLQA[R-$^{13}C_6$]	
CFTR04	Native	NSILNPINSIR	$[M + 2H]^{2+} \rightarrow y_8, y_7, y_6$
R(Δ6)-CFTR04	SILAC	NSILNPINSI[R-$^{13}C_6$]	
L(Δ2)-CFTR04	SILAC	NSI[L-1,2-$^{13}C_2$]NPINSIR	

[a] Δx denotes a mass increase, i.e., Δ4 means a mass increase of 4 Da for the isotope-labeled peptide compared with the native counterpart.

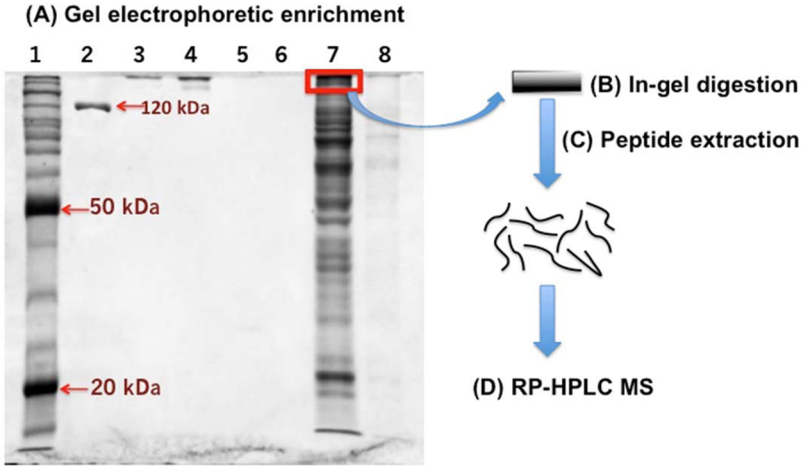

Figure 1. Gel electrophoretic enrichment (GEE) of large membrane proteins. The large proteins are sampled by slicing the gel piece at the interface of the stacking gel and the resolving gel (A). Samples loaded are as follows: 1, protein ladder; 2, β-galactosidase; 3, 500 ng of CFTR; 4, 1 μg of CFTR; **5**, 10 μg of BHK-wtCFTR biotinylated fraction; 6, 1 μg of BHK-wtCFTR biotinylated fraction; 7, 30 μg of BHK-wtCFTR Triton X-100 extract; and 8, 3 μg of BHK-wtCFTR Triton X-100 extract. The gel piece (red box) was cut for in-gel digestion (B; other bands were relatively low-molecular-weight membrane proteins removed by GEE), and the resulting peptides were extracted (C) and analyzed by LC−SID−MRM MS (D).

Chadds Ford, PA), and the length of resin bed was around 15 cm. Solvent A was composed of 98.8% H_2O, 1.0% acetonitrile, and 0.2% formic acid (v/v), and solvent B was composed of 98.8% acetonitrile, 1.0% water, and 0.2% formic acid (v/v). A typical running gradient was 1% B at 0 min → 3% B at 5 min → 30% B at 50 min → 80% B at 59 min → 90% B at 69 min → 1% B at 80 min, with a 10 min equilibration. Samples were loaded on the trap column at flow rate of 4 μL/min at 1% solvent B for 10 min. A triple quadruple mass spectrometer was used, 4000 QTrap from ABSCIEX (Foster City, CA). Transitions for the native and stable isotope labeled peptides are summarized in Table 1.

2.7. Peptide Derivatization and Ultrathroughput Multiple Reaction Monitoring (uMRM) MS[34]

Following in-gel digestion of the HEK293F-wtCFTR cell lysate, the N-termini of the peptide mixtures were derivatized with anhydrides (acetic, propionic, butyric, valeric, or succinic anhydride). To each sample were added 70 μL of acetonitrile, 70 μL of 1 M ammonium bicarbonate, and 10 μL of the appropriate anhydride, and samples were incubated on ice for 1 h. Following the incubation, another addition of 100 μL of acetonitrile, 100 μL of 1 M ammonium bicarbonate, and 10 μL of the appropriate anhydrate were added. Samples were then incubated on ice for an additional 2 h. After the chemical derivatization, the modified peptides were dried and desalted using hydrophilic−lipophilic-balanced sorbent (HLB, Oasis) and lyophilized. The resulting five derivatized digests were combined for a single uMRM MS experiment.

2.8. PM CFTR Turnover Study

In a typical experiment, BHK-wtCFTR cells for each time point were grown in triplicate in nonisotopic media. At time zero, the media was aspirated, the cells were washed twice with PBS, and isotopically labeled media was added as follows: (1) BHK-wtCFTR media minus arginine, leucine, and lysine and supplemented with 0.4 mM L-arginine·HCl, 0.8 mM L-lysine·HCl, and 0.8 mM L-leucine-1,2-$^{13}C_2$ or (2) BHK-wtCFTR media minus arginine, leucine, and lysine and supplemented with 0.4 mM L-arginine-$^{13}C_6$·HCl (Cambridge Isotopes, Andover, MA), 0.8 mM L-lysine-$^{13}C_6{}^{15}N_2$·HCl (Cambridge Isotopes, Andover, MA), and 0.8 mM L-leucine. At the appropriate time, the cells were biotinylated, combined, and fractionated as previously detailed. After protein digestion, signature peptides for native and stable isotope labeled CFTR were quantified by LC−SID−MRM MS.

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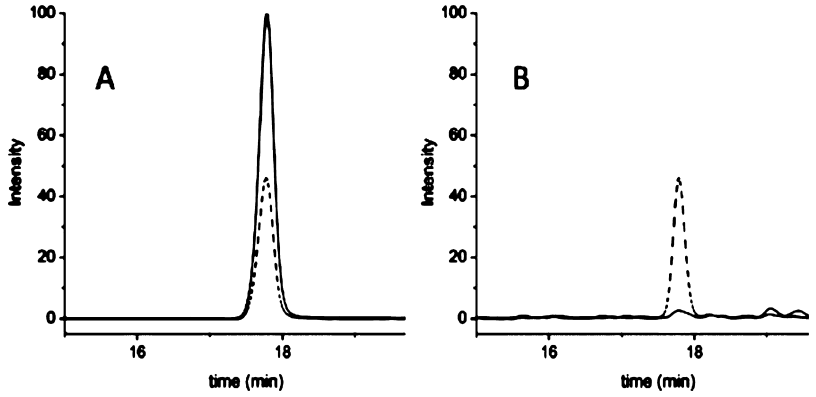

Figure 2. It is essential to use a tandem enrichment strategy for preparing PM CFTR samples. MRM ion chromatograms are shown for the native signature peptide (solid line) and the spiked stable isotope reference peptide (dotted line) of digests of HT-29 cell lysate prepared by surface biotinylation and GEE (A) and surface biotinylation only (B).

3. RESULTS AND DISCUSSION

3.1. General Strategy for Cell-Surface Biotinylation and GEE for the Tandem Enrichment of Large and Low-Abundance PM Proteins

Membrane proteins are critical for cells to communicate with the extra-cellular environment. Receptors and transporters in the PM thus form the most dominant protein class for developing drugs to treat human diseases.[35] These membrane proteins are typically large, hydrophobic, and low in abundance. Preparation of membrane proteins and proteome samples is a major analytical bottleneck in analysis.[19,20] We have thus designed a novel, broadly applicable workflow to enrich low-abundance, large membrane proteins by sequential use of (1) the established method of cell surface biotinylation and (2) a method of gel electrophoretic enrichment (GEE), which is a variant of gel electrophoresis (Scheme 1). In this work, we have established a workflow for enriching PM CFTR and evaluated experimental variations in major steps of the workflow (see Supporting Information Text S1, Figure S1, and Tables S1 and S2).

GEE makes special use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation of protein mixtures. In the GEE method, nongradient gels are used for protein fractionation, and the gel compositions are formulated in such a way that proteins larger than a minimum molecular weight do not migrate appreciably in the resolving gel. It is a simple but effective approach for enriching high-molecular-weight proteins (e.g., >150 kDa) in a complex mixture (Figure 1). For enriching CFTR, the composition for the resolving gel is 8% acrylamide and 0.2% bis(acrylamide). This composition slows the migration of large proteins and limits their location to the interface region of the resolving and stacking gel while allowing for the electrophoretic migration of smaller proteins. With or without gel staining, a protein fraction with a predictable lower cutoff of molecular weight (i.e., proteins that have molecular weights higher than a designed value) can be reproducibly sampled by excising gel slices at a fixed location near the interface (Figure 1) for subsequent in-gel digestion and MS analysis.

Expression of CFTR in the PM is low, and the tandem enrichment strategy made CFTR quantitation possible by LC–SID–MRM MS,[36] without using antibody enrichment of the protein;[25] this is due to both the increase in CFTR concentration and the decrease in the sample complexity for MS quantitation. Mutant CFTR proteins, even after therapeutic intervention, exist in even lower amounts in the PM; thus, enrichment of PM CFTR is necessary for quantifying the protein by MS. A study was performed to compare LC–SID–MRM MS quantitation of CFTR signature peptides prepared by the tandem enrichment workflow with those in the direct digestion mixture of the PM subproteome prepared by surface biotinylation and avidin pull-down for HT-29 cells. Only the former workflow resulted in confident quantitation, and the measurement gave 40 fmol of apical PM CFTR per million cells, equivalent to 23 000 molecules per cell (Figures 1 and 2). It should be noted that this amount is based on a peptide quantitation reference standard [^{18}O(Δ4)-CFTR01][25] and thus represents the lower range of PM CFTR in HT-29 cells (see later for a discussion on protein vs peptide quantitation reference standards). Although the GEE method by itself can be used for preparing CFTR signature peptides from various protein (mixture) samples containing CFTR, it is necessary to have the surface biotinylation enrichment in the workflow. Thus, only matured full-length CFTR (commonly referred to as Band C[37] by the CF research and clinical community) in the PM is sampled for quantitation; the GEE method does not separate the nonglycosylated CFTR (commonly referred to as Band B[37]) from that which is fully matured.

Another analytical advantage for the GEE method is compatibility with various solubilizing reagents used for preparing samples containing CFTR. To enhance solubilization of membrane proteins, SDS and other detergents are used in the extraction and digestion buffers. However, the presence of detergents can interfere with protease activity during protein digestion and can also affect chromatographic separation and suppress ionization of resulting peptides. Although the detergents can be removed after digestion, cleanup of detergent-containing peptide mixtures can cause significant sample loss and has varying efficiencies. In contrast, during the gel-based sample preparations, detergents and other interfering reagents are removed by extensive washing before digestion. It has been reported that protein mixtures can be concentrated to a thin band after a short-running SDS-PAGE; the main purpose of this practice has been the removal of detergents in membrane protein preparations for enhanced MS-based proteomic analysis,[38,39] not to enrich low-abundance proteins based on their size. Compared to the in-solution digestion, the in-gel digestion also increases the digestion efficiency for membrane proteins;[40,41] this is possibly attributed to the

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Table 2. Preparation of the SILAC CFTR Quantitation Reference Standard[a]

	area CFTR01		area CFTR02		area ratio CFTR01	area ratio CFTR02	
sample no.	native	L(Δ4)	native	L(Δ6)	native/L(Δ4)	native/L(Δ6)	SILAC CFTR amount (ng)
1	4457	2100	10 080	4703	2.12	2.14	140.8
2	8690	3980	18 850	9263	2.18	2.03	142.9
3	3890	1810	7879	3717	2.15	2.12	140.8
						average	141.5

[a]The area ratios between the native peptides and heavy isotope labeled peptides were used to calculate the concentration of SILAC CFTR in cell lysate, which was labeled with leucine-1,2-^{13}C. Highly-purified full-length CFTR (300 ng) was used to calibrate the absolute amount of SILAC CFTR. Sample volume was 30 μL.

Table 3. Absolute Amounts of PM CFTR for HEK293F-wtCFTR Cells[a]

	relative area CFTR01			area ratio		
doxycycline (μg/mL)	native	L(Δ4)[b]	L(Δ7)[c]	native/L(Δ4)	native CFTR absolute amount (ng)[d]	CFTR digestion efficiency (%)[e]
0	0	3802	16 350	0	0	
0.25	3654	4229	15 640	0.86	130	15
0.75	4405	2110	7337	2.09	313	16
1.00	3279	1103	3723	2.97	446	17
1.25	29 880	7560	30 190	3.95	593	14

[a]Area ratios (native/SILAC) were used to calculate expressed native CFTR. [b]150 ng of SILAC CFTR was added to each sample. [c]500 fmol of L(Δ7)-CFTR01 was added. [d]Amount of PM CFTR for each 10 cm plate. [e]Relative areas for L(Δ4) and L(Δ7) peptides, together with the known amounts of SILAC CFTR and L(Δ7)-CFTR01, gave measurements for the digestion efficiency for CFTR. These numbers should represent the lower limits for the digestion efficiency because sample loss during GEE and peptide preparation could also be attributed to the calculated efficiency.

decreased protein aggregation during digestion. By immobilizing hydrophobic membrane proteins in the gel matrix, aggregation of membrane proteins and large protein fragments produced at the initial stage of the protein digestion is likely to be minimized.

3.2. SILAC CFTR as a Quantitation Reference Standard for the Absolute Quantitation of PM CFTR

The full-length CFTR quantitation reference standard was prepared through metabolic labeling using the SILAC method.[31] Lysates of BHK-wtCFTR cells cultured with media containing leucine-1,2-^{13}C CFTR (seven doublings) were prepared by a buffer containing Triton X-100. The SILAC CFTR amount in the Triton X-100 extract was quantified against a highly purified, full-length native CFTR standard (this native CFTR could be used as the master reference standard for different laboratories) using the GEE method for sample preparation and LC−SID−MRM MS for CFTR quantitation. Concentration of native CFTR was determined by amino acid analysis to be 0.175 (±0.025) μg/μL. The quantity of SILAC CFTR was measured in triplicate using two signature peptides (CFTR01 and CFTR02). The protein concentration in the SILAC Triton X-100 extract was determined to be 4.72 (±0.07) ng/μL (Table 2). It should be noted that the SILAC CFTR reference standard was used as a Triton X-100 extract directly for quantifying CFTR samples.

A quantitative comparison was performed to examine the difference in CFTR between using the SILAC CFTR protein standard and using a stable isotopic synthetic peptide, L(Δ7)-CFTR01. HEK293F-wtCFTR cells, which overexpress wtCFTR upon induction with doxycycline at different concentrations (0, 0.25, 0.50, 0.75, and 1.0 μg/mL), were used. Apical PM CFTR was prepared by the tandem enrichment workflow (Scheme 1). One additional quantitation reference, peptide L(Δ7)-CFTR01, was added to the peptide mixtures resulting from in-gel digestion. Furthermore, five different samples were derivatized with different acid anhydrates (acetic, propionic, butyric, valeric, or succinic anhydride); with this sample-specific coding procedure, all five samples were quantified in a single LC−SID−uMRM MS experiment.[34] The signature peptide CFTR01 was monitored in this study (Table 3; also see Supporting Information Figures S2 and S3). Expression of native CFTR in these doxycycline-inducible HEK293F-wtCFTR cells was determined according to both L(Δ4)-CFTR01 and L(Δ7)-CFTR01. While both data sets observed increased expression of CFTR, with the increase in the doxycycline concentration, CFTR amounts measured according to the peptide standard L(Δ7)-CFTR01 (added after in-gel-digestion of CFTR) were only 14−17% of the corresponding measurements based on L(Δ4)-CFTR01, produced via in-gel digestion of SILAC CFTR (Table 3). This study shows that for absolute quantitation of membrane proteins like CFTR, it is essential to use protein standards to obtain better quantitation accuracy.

The use of an isotopic protein reference allows for advantageous addition of an internal standard at an early step of sample preparation. For the PM CFTR quantitation, SILAC CFTR was added as the reference in the surface biotinylation fraction of membrane proteins before further sample preparations. In comparison, conventional LC−SID−MRM MS uses isotopic reference peptides.[25−28,42] For absolute quantitation of membrane proteins, peptide references are not acceptable. Membrane proteins are hard to digest; in a typical experiment, the yield of signature peptide CFTR01 is only 14−17%, and this yield varies from one sample preparation to another (Table 3). Furthermore, CFTR is prone to aggregation and degradation during sample preparation; for instance, preparation of CFTR for SDS-PAGE is performed at 30 °C instead of boiling temperature. Addition of SILAC CFTR before GEE and protein digestion largely minimized variations in sample preparation and afforded the absolute quantitation of CFTR in CFBE cells.

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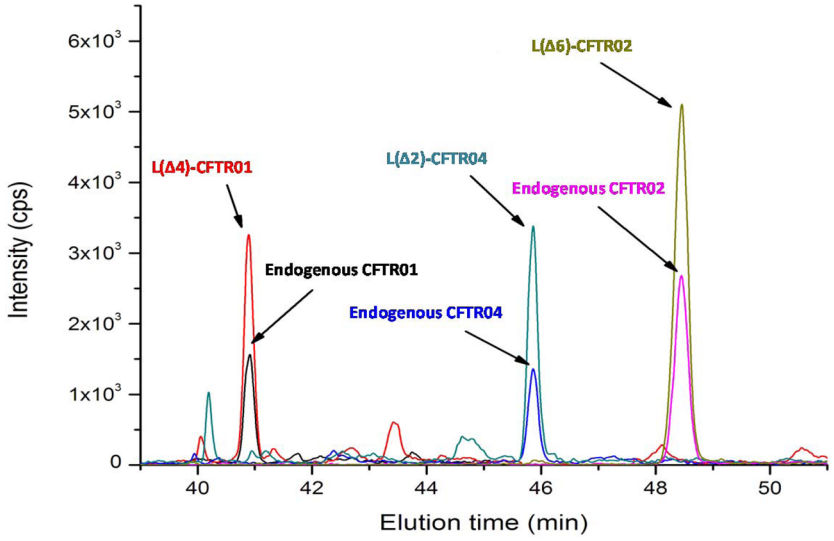

Figure 3. LC−SID−MRM MS chromatograms of signature peptides for quantifying CFTR in the apical PM of CFBE cells grown on filters. Peptides are denoted as in Table 1.

3.3. Absolute Quantitation of Apical PM Expression of CFTR in CFBE 41o- Cells

The CFBE cell line is a common model for CF research and drug development. We performed absolute quantitation of apical PM CFTR in CFBE 41o- cells grown on plates and snapwell filters via LC−MRM MS of signature peptides. Three signature peptides, CFTR01, CFTR02, and CFTR04, were monitored by LC−SID−MRM MS (Figure 3), but only CFTR01 and CFTR02 peptides were consistently able to be quantified for measuring their precursor CFTR protein in the PM together with CFTR in the cytoplasm (i.e., CFTR in the Triton X-100 extract after removing PM CFTR). Quantitation results based on CFTR01 and CFTR02 were comparable (Table 2). Each sample was repeated with at least three different biological preparations. Results are summarized in Table 4. As expected, surface expression of CFTR in CFBE cells (14.1% of CFTR in the whole cell lysate) grown in snapwell filters is higher than that of CFBE cells grown in flasks (10.1%). This is in agreement with semiquantitative western blot analysis (Supporting Information Figure S4). CFBE cells grown on snapwell filters mimic airway epithelial cells, with the air−liquid interface creating a polarized cell environment, increasing the PM expression of CFTR. The absolute quantitation method reported in this work will also enable accurate measurements of CFTR in primary cells. Absolute quantitation of PM CFTR will set the basis for the comparison of results and thus will greatly amplify the overall outcome of CF research and therapy. In reference, high reproducibility for LC−SID−MRM MS quantitation of protein targets in complex matrices has recently been shown for sample-to-sample, day-to-day, analyst-to-analyst, and lab-to-lab comparisons.[26]

Table 4. Absolute Quantitation and Surface Expression of CFTR Protein in CFBE 41o- Cells

sample	CFTR amount (ng/10^6 cells)	PM expression (%)
plate, biotinylated	2.90 ± 0.03	10.3 ± 0.4
plate, flow-through	25.0 ± 0.8	
filter, biotinylated	6.75 ± 0.18	14.1 ± 2.0
filter, flow-through	41.2 ± 4.7	

3.4. Turnover of CFTR in the Apical PM

We designed and performed the first quantitative analysis of CFTR turnover in the PM using stable isotopes. BHK-wtCFTR cells were originally cultured in native culture media. At time zero, culture media were switched to SILAC media containing stable isotope labeled lysine and arginine or leucine; thus, native CFTR in the PM started to decrease with time, and SILAC CFTR labeled with the stable isotopes started to appear and increase. At different time intervals, cells were harvested, and the protein fraction containing PM CFTR was processed according to the tandem enrichment workflow (Scheme 1). Native CFTR in the PM was quantified against the newly synthesized isotopic CFTR by LC−SID−MRM MS of signature peptides. The time course for the newly produced CFTR was followed for up to 60 or 84 h, through two experiments. The average area ratio of the signature peptides from the newly incorporated CFTR versus the native CFTR (isotopic/native) was normalized to the maximum, and then the percentage of remaining native CFTR (denoted as R in Figure 4) was calculated. The natural logarithm of R (for data points up to 48 h; incorporation of isotope labels were saturated beyond 48 h) was plotted against time and fitted according to first-order kinetics[11,43] with an adjusted R^2 of 0.9496. The half-life ($t_{1/2}$) of native CFTR in the PM was calculated to be 29.0 ± 2.5 h.

Rescuing CFTR mutant proteins for expression in the apical PM is only the first step. The rescued mutants have to be stable in the membrane and perform the channel function for Cl^- ions. It has been recently reported that the CF drug VX-809 can fully rescue the PM expression of F508del, but the lifespan of the rescued mutant falls short compared with that of wtCFTR.[11] Conventional western blot analysis of CFTR cannot be applied for turnover studies of PM CFTR. Although metabolic pulse−chase of radioisotopes provides a useful tool for this analysis,[11] a more convenient method for accurate measurements of the protein turnover in the PM can be greatly beneficial to CF research and drug development. The combination of metabolic stable isotope labeling and quantitative proteomics is an emerging alternative to radioisotope methods.[44,45] Under this experimental framework, coupling the tandem enrichment of CFTR with highly sensitive

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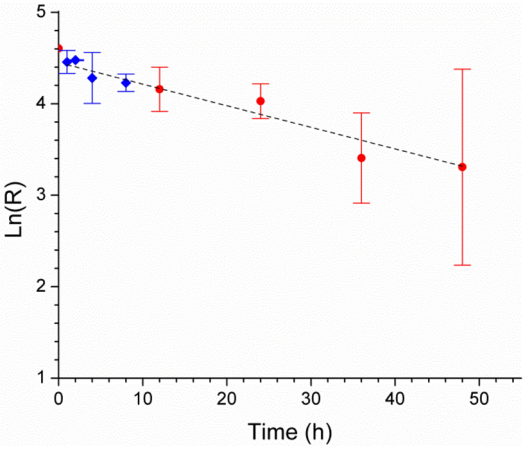

Figure 4. Turnover of CFTR in the PM of BHK-wtCFTR cells. R represents normalized percentage degradation of native CFTR (see Results and Discussion). Blue and red data points are from two separate sets of experiments: isotopic leucine and isotopic arginine/lysine CFTR incorporation, respectively. Peptides CFTR01, 03, and 04 were monitored for the blue data points, and the individual transitions were used for the error calculations. Peptides CFTR01–04 were monitored for the red data points, and analytical triplicate measurements were used for error calculations [slope = −0.0239 ± 0.0021; adjusted R^2 = 0.9496].

MRM MS has made it possible to measure CFTR turnover ($t_{1/2}$ = 29.0 ± 2.5 h) in the PM of BHK-wtCFTR cells. In comparison, a shorter half-life ($t_{1/2}$ ~14 h) was recently reported for CFTR in the whole-cell lysate of the same cell line.[11] These results are in agreement with the observation that PM proteins degrade more slowly than proteins that do not reach the membrane.[43]

4. CONCLUSIONS

Membrane receptors and transporters form the most important class of protein targets for understanding intercellular communication and developing new drugs. Recent advances in MS-based quantitative proteomics, in combination with new sample preparation workflows, have made it possible to obtain unprecedented accuracy and precision in membrane protein measurements. Targeted quantitation of apical PM CFTR reported in this work exemplifies an important application of contemporary proteomics technologies, and it will significantly help with understanding the disease mechanism of CF and developing future medicine for its treatment.

■ ASSOCIATED CONTENT

Supporting Information

Text S1: Method development using CFTR-overexpressing BHK cells. Figure S1: Experimental design for examining the assay reproducibility. Figure S2: Quantitation of CFTR protein in the apical plasma membrane of HEK293F-wtCFTR cells upon doxycycline induction through multiplexing technology. Figure S3: Fluorescent microscopy of HEK293F-wtCFTR cells upon doxycycline induction at different concentrations for producing wtCFTR. Figure S4: Western blot analysis of CFTR in the apical plasma membrane and flow-through (the unbound fractions upon avidin enrichment of surface biotinylated CFTR in Triton X-100 extracts). Table S1: CFTR quantitation in BHK cells with various sample treatment methods. Table S2: Relative error in CFTR quantitation in various levels of sample treatment for BHK-wtCFTR cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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