Activation of 3-Phosphoinositide-dependent Kinase 1 (PDK1) and Serum- and Glucocorticoid-induced Protein Kinase 1 (SGK1) by Short-chain Sphingolipid C4-ceramide Rescues the Trafficking Defect of ΔF508-Cystic Fibrosis Transmembrane Conductance Regulator (ΔF508-CFTR)*

Received for publication, July 22, 2014, and in revised form, November 7, 2014. Published, JBC Papers in Press, November 10, 2014, DOI 10.1074/jbc.M114.598649

Hung Caohuy§, Qingfeng Yang‡, Yvonne Eudy‡, Thien-An Ha‡, Andrew E. Xu‡, Matthew Glover‡, Raymond A. Frizzell§, Catherine Jozwik‡, and Harvey B. Pollard§,‡

From the §Department of Anatomy, Physiology, and Genetics, School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814 and the ‡Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15224

Background: The discovery of small molecules to correct the trafficking defect of the mutant ΔF508-CFTR protein has been challenging.

Results: C4-ceramide rescues and stabilizes ΔF508-CFTR. High basal secretion of interleukin-8 is also suppressed.

Conclusion: Results identify a novel mechanism by which C4-ceramide activates the PDK1/SGK1 pathway, thereby rescuing ΔF508-CFTR.

Significance: C4-ceramide may be a novel candidate therapeutic for CF patients.

Cystic fibrosis (CF) is due to a folding defect in the CF transmembrane conductance regulator (CFTR) protein. The most common mutation, ΔF508, prevents CFTR from trafficking to the apical plasma membrane. Here we show that activation of the PDK1/SGK1 signaling pathway with C4-ceramide (C4-CER), a non-toxic small molecule, functionally corrects the trafficking defect in both cultured CF cells and primary epithelial cell explants from CF patients. The mechanism of C4-CER action involves a series of mutual autophosphorylation and phosphorylation events between PDK1 and SGK1. Detailed mechanistic studies indicate that C4-CER initially induces autophosphorylation of SGK1 at Ser422. SGK1[Ser(P)422] and C4-CER coincidently bind PDK1 and permit PDK1 to autophosphorylate at Ser241. Then PDK1[Ser(P)241] phosphorylates SGK1[Ser(P)422] at Thr256 to generate fully activated SGK1[Ser422, Thr(P)256]. SGK1[Ser(P)422, Thr(P)256] phosphorylates and inactivates the E3 ubiquitin ligase Nedd4-2. ΔF508-CFTR is thus free to traffic to the plasma membrane. Importantly, C4-CER-mediated activation of both PDK1 and SGK1 is independent of the PI3K/Akt/mammalian target of rapamycin signaling pathway. Physiologically, C4-CER significantly increases maturation and stability of ΔF508-CFTR (t½ ≈ 10 h), enhances cAMP-activated chloride secretion, and suppresses hypersecretion of interleukin-8 (IL-8). We suggest that candidate drugs for CF directed against the PDK1/SGK1 signaling pathway, such as C4-CER, provide a novel therapeutic strategy for a life-limiting disorder that affects one child, on average, each day.

Cystic fibrosis (CF) is a life-limiting, autosomal recessive disease, which is due to mutations in the CF transmembrane conductance regulator (CFTR) gene. The most common of these mutations is ΔF508, which results in failure of the mutant protein to fold correctly and thus to traffic efficiently to the apical plasma membrane in epithelial cells from the lungs, pancreas, and other organs (1). At the plasma membrane, wild-type CFTR forms a cAMP-activated chloride channel (2) and suppresses secretion of IL-8 (3). Instead, ΔF508-CFTR is retained in the endoplasmic reticulum, where it is subsequently degraded by the proteasomal system (4). Importantly, when ΔF508-CFTR is permitted to traffic to the plasma membrane, both cAMP-activated chloride channel activity and anti-inflammatory functions are rescued (5–7). However, despite traf...
Rescue of ΔF508-CFTR Trafficking by C4-ceramide

ficking rescue by corrector treatment or low temperature (27 °C) incubation, rescued ΔF508-CFTR exhibits significantly reduced plasma membrane density (8). For these reasons, strategies for CF therapy have focused on the development of small molecules that not only rescue the trafficking defect but also enhance the stability of ΔF508-CFTR at the plasma membrane (9).

We previously reported that the glucocorticoid dexamethasone (DEX) functionally rescues the ΔF508-CFTR trafficking defect in CF lung and pancreas epithelial cells (10). Such rescue depends upon the coincident activation of 3-phosphoinositide-dependent kinase 1 (PDK1) by phosphoinositide-3-kinase (PI3K) and of serum/glucocorticoid-induced protein kinase 1 (SGK1) by DEX. Activation of PDK1/SGK1 results in phosphorylation of neural precursor cell-expressed, developmentally down-regulated 4-2 (Nedd4-2), which inactivates Nedd4-2. Nedd4-2 inactivation results in decreased ubiquitination of mutant CFTR, thereby preventing its degradation and promoting trafficking to the plasma membrane. However, two intrinsic problems limit this specific approach as a therapeutic strategy for CF. First, glucocorticoids have many off-target effects; second, activation of PI3K is intrinsically proinflammatory (11) and carcinogenic (12). Alternatively, we reasoned that it would be attractive to discover a chemical agent that could not only activate PDK1, independently of PI3K activity, but also activate SGK1, independently of DEX, and still inactivate Nedd4-2.

The activation of SGK1 by PDK1 is typically driven by PI3K-dependent synthesis of phosphatidylinositol 3,4,5-triphosphate (13, 14). However, the yeast homologues of PDK1 and SGK1 (Pkh1 and Ypk1, respectively) do not require phosphoinositides for Ypk1 activation (15). Instead, activation of Ypk1 by Pkh1 depends upon other types of agents, including sphingolipids (16). Therefore, we hypothesized that sphingolipid signaling in mammals might also activate PDK1 and drive SGK1/Nedd4-2-mediated rescue of ΔF508-CFTR without direct activation of PI3K and glucocorticoid receptor signaling. Here, we show that functional rescue of ΔF508-CFTR can be achieved with short-chain ceramides (CERs), the most effective of which is C4-CER. C4-CER-mediated rescue requires both PDK1 and SGK1 but is totally independent of the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway. These data suggest that C4-CER may be a candidate therapeutic for CF, specifically by rescuing the most common mutation, ΔF508-CFTR, from proteasomal destruction.

EXPERIMENTAL PROCEDURES

Ceramides and Antibodies—The following sphingolipid CERs were purchased from Avanti Polar Lipids Inc.: d-erythro-sphingosine (d18:1; SPH), sphingosine 1-phosphate (SHP-1-P), N-acetyl-n-erythro-sphingosine (d18:1/2α; C2-CER), N-butyro-d-erythro-sphingosine (C4-CER), N-hexanoyl-d-erythro-sphingosine (C6-CER), N-octanoyl-d-erythro-sphingosine (C8-CER), N-lauroyl-d-erythro-sphingosine (C12-CER), N-palmitoyl-d-erythro-sphingosine (C16-CER), N-stearoyl-d-erythro-sphingosine (C18-CER), N-behenoyl-d-erythro-sphingosine (C22-CER), d-glucosyl-β,1–4-N-stearoyl-d-erythro-sphingosine (C18-GlcCER), N-lignoceryl-ceramide-1-phosphate (C24-CER-1-P), N-(6-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)hexanoyl)-d-erythro-sphingosine (NBD-C6-CER), and N-(18-((7-nitro-2–1,3-benzoxadiazol-4-yl)amino)stearyl)-d-erythro-sphingosine (NBD-C18-CER). The following C4-CER analogs were synthesized by Toronto Research Chemicals Inc.: N-butyroxyphosphatidoglycerol (C4-phytoCER), N-butanoyl-d-erythro-sphingosine-1-phosphate (C4-CER-1-P), (R,R,S)-N-(1-((β-d-glucopyranosylxy)methyl)-2-hydroxy-3-heptadecenyl) butanamide (C4-GlcCER), C4-CER analog 1 (C4-CER-A1; 14:1/4:0), C4-CER analog 2 (C4-CER-A2; d10:1/4:0), and C4-CER analog 3 (C4-CER-A3; d7:1/4:0).

The following antibodies were obtained commercially: rabbit anti-P13K p110 subunit α or β (ab40776 or ab32569, Abcam), mouse anti-PDK1 (611071, BD Transduction Laboratories), rabbit anti-SGK1 (ab43606, Abcam), mouse anti-Lamin A/C (612163, BD Transduction Laboratories), mouse Nedd4-2 (ab58093, Abcam), rabbit NDRG1 (1-N-Myc down-regulated gene 1; 5196, Cell Signaling), mouse anti-β-actin (A5441, Sigma-Aldrich), mouse anti-CFTR C terminus (24-1, R&D Systems), rabbit anti-Akt (9272, Cell Signaling), rabbit anti-Rictor (2140, Cell Signaling), and mouse anti-ubiquitin P4D1 (sc-8017, Santa Cruz Biotechnology, Inc.) antibody. To detect phosphorylated proteins, the following phospho-specific antibodies were used: mouse anti-PDK1 at Ser241 (558395, BD Pharmingen), rabbit anti-SGK1 at Ser422 (ab55281, Abcam), goat anti-SGK1 at Thr256 (sc-16744, Santa Cruz Biotechnology), rabbit anti-Akt at Ser473 and Thr308 (3787 and 2965, Cell Signaling), rabbit anti-mouse Nedd4-2 at Ser328 (corresponding to human Ser460; ab95399, Abcam), rabbit anti-NDRG1 at Thr346 and Ser330 (3217 and 3506, Cell Signaling), and rabbit anti-phospho-Ser/Thr-phospho-Thr (ab17464, Abcam) antibody.

Cell Cultures—CFPAC-1 (ΔF508) and wild-type CFTR-repaired CFPAC-1 pLj6 cells were maintained as described previously (10). Wild-type or ΔF508-CFTR-transduced CFBE41o cells (HBE or CFBE, respectively) were generous gifts from Dr. Dieter Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA) and were grown in minimum Eagle’s medium supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 10% FBS, and 1 µg/ml basicidaline (HBE) or 2 µg/ml puromycin (CFBE). Human colon T84 cells, which express wild-type CFTR endogenously, were obtained from the American Type Culture Collection and were grown according to the manufacturer’s instructions. All cells were maintained in a 5% CO₂, 95% air incubator at 37 °C.

Chemical Treatments—All experiments were performed at 37°C. For experiments using short- and/or long-chain CERs, all lipids were prepared in a solvent mixture containing ETOH and dodecane (49:1 v/v; Sigma-Aldrich). This solvent mixture has been well documented as an efficient delivery vehicle for exogenous long-chain CERs (17–25). For experiments using only C4-CER and its analogues, ETOH was used as a delivery vehicle. The final ETOH/dodecane or ETOH concentration used was 0.1% or less. CFPAC-1, repaired CFPAC-1 pLj6, and T84 cells were treated for 20 h with a 10 µM concentration of different CER compounds or an equivalent amount of vehicle. For dose- and time-dependent experiments, CFPAC-1 or CF bronchial epithelial (CFBE) cells were treated for 20 h with vehicle or various concentrations (1–20 µM) of C4-CER, inactive C4-di-hydroceramide (C4-dCER), or VX-809 (Selleckchem) or incubated with 10 µM C4-CER for 1, 4, 8, and 20 h. For testing
selected CER species, CFPAC-1 or CFBE cells were incubated for 20 h with indicated concentrations of SPH, SPH-1-P, C24-CER-1-P, C18-Glc-CER, C4-CER-1-P, C4-Glc-CER, C4-phyto-CER, or C4-CER analogs with a truncated sphingoid backbone (i.e. C4-CER A1, A2, or A3).

For blockade of CER metabolic pathways, CFPAC-1 cells were pretreated for 4 h with 10 μM MAPP (Biomol) (26), 2 μM DMS (Enzo Life Sciences) (27), 20 μM fumonisin B1 (Sigma-Aldrich) (28), 10 μM D-PDMP (Biomol) (29), or 1 μM NVP-231 (Sigma-Aldrich) (30), followed by a 20-h incubation with vehicle or 10 μM C4-CER in the presence or absence of the above CER metabolic pathway inhibitors. For inhibition of PI3K kinase activity, CFPAC-1 cells were pretreated for 4 h with 0.2 μM wortmannin (Calbiochem) (31), 8 μM LY294002 (Calbiochem) (32), or 2 μM PI-103 (Tocris Biosciences) (33), followed by a 20-h incubation with vehicle or 10 μM C4-CER in the presence or absence of the above PI3K inhibitors. To block PDK1 or SGK1 kinase activity, the cells were pretreated for 4 h with various concentrations of PDK1 inhibitor GSK2334470 (Tocris Biosciences) (34) or SGK1 inhibitor GSK650394 (Tocris Biosciences) (35), followed by a 20-h incubation with vehicle or 10 μM C4-CER in the presence or absence of various concentrations of GSK2334470 or GSK650394, respectively. All chemical inhibitors were prepared in ETOH or DMSO. After treatments, cells were subjected to cell lysis or cell surface biotinylation, followed by Western blot analysis.

Cycloheximide Chase—CFPAC-1 or CFBE cells were pretreated at 37 °C for 20 h with C4-CER or were grown at 27 °C for 48 h, to induce maturation of ΔF508-CFTR. Pretreated cells were then incubated at 37 °C with 20 μg/ml cycloheximide (CHX; Sigma-Aldrich) for the indicated times (0, 2, 4, and 8 h). At the end of each indicated time period, cells were subjected to cell lysis or cell surface biotinylation, followed by Western blot analysis.

siRNA Transfection—The following SMART-pool siRNAs were obtained from Ambion: PI3K catalytic subunits (p110) α and β, PDK1, SGK1, Rictor, Lamin A/C, and scrambled control. Transfection of all siRNAs (50 nM) to CFPAC-1 cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were then grown at 37 °C for 48 h in culture medium, followed by a 20-h treatment with vehicle or 10 μM C4-CER. The cells were then subjected to cell lysis or cell surface biotinylation, followed by Western blot analysis.

Cell Surface Biotinylation Assay—Chemically treated or siRNA-transfected cells were rapidly washed with cold PBS (pH 8.2) solution supplemented with 1 mM CaCl2 and 1 mM MgCl2 and then subjected to cell surface biotinylation using sulfo-NHS-SS-biotin (Pierce) as described previously (10). After cell lysis, biotinylated proteins from the cell lysates were pulled down using streptavidin-agarose (Pierce) and were eluted in Laemmli SDS-PAGE sample buffer supplemented with 50 mM dithiothreitol, followed by SDS-PAGE and Western blot analysis.

Cell Lysis and Western Blotting—Cells were lysed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, 1% (v/v) SDS) supplemented with Halt™ protease/phosphatase inhibitors (Pierce), followed by sonication and centrifugation at 14,000 × g for 10 min at 4 °C. Protein concentrations were determined according to the BCA method (Pierce). Equal amounts of protein from the cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked in Tris-phosphate saline buffer containing 5% (w/v) dry milk and 0.5% (v/v) Tween 20. After incubation with the primary antibody overnight at 4 °C, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch). Immunoreactive proteins were detected using Immobilon enhanced chemiluminescence (ECL; Millipore), and the signals were captured by a FujiFilm LAS-1000 system and quantitated by FujiFilm Image Gauge version 3.0. The quantitated values of CFTR band B and band C or surface CFTR were normalized to the corresponding values of β-actin detected in the same cell lysates.

CFTR Immunoprecipitation—Equal amounts of total protein from the cell lysates were precleared with protein A-immobilized Dynal beads (Invitrogen). The resulting lysates were then incubated with mouse anti-CFTR C-terminal antibody for 4 h, followed by overnight incubation at 4 °C with protein A-immobilized Dynal beads. IgG from the same species as the antibody being used for the immunoprecipitation was used as a negative control. Immunoprecipitated proteins were eluted in Laemmli SDS-PAGE sample buffer and separated by SDS-PAGE, followed by Western blot analysis.

In Vitro Phosphorylation of CFTR—Wild-type and ΔF508-CFTR immunoprecipitates from untreated repaired and CFPAC-1 cell lysates, respectively, were washed with phosphorylation buffer (50 mM Tris, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol) and then resuspended in 30 μl of the same buffer containing 100 ng of purified recombinant human S422D-SGK1 mutant protein (Millipore) and 1 × Mg2+-ATP mixture (Millipore). The reactions were incubated at 30 °C for 1 h in the presence or absence of 100 ng of active recombinant human PDK1 (Millipore), stopped in Laemmli SDS-PAGE sample buffer, followed by SDS-PAGE. CFTR phosphorylation was immunodetected using an anti-Thr(P)/Ser(P) antibody.

In Vitro Phosphorylation of SGK1 and PDK1—For detecting phosphorylation of SGK1 at Ser422, purified recombinant human SGK1 (100 ng; Abcam) was incubated at 30 °C for 1 h in phosphorylation buffer containing C4-CER (1, 5, and 10 μM), 10 μM C4-dCER, or vehicle in the presence or absence of 1 × Mg2+-ATP mixture. For detecting phosphorylation of SGK1 at Thr256, SGK1 (100 ng) and active recombinant human PDK1 (100 ng; Abnova) were incubated at 30 °C for 1 h with vehicle or 5 μM C4-CER, C4-dCER, or C22-CER. Additionally, to determine whether C4-CER further enhances Ser241 autophosphorylation of PDK1, PDK1 (100 ng) was incubated at 30 °C for 1 h in phosphorylation buffer containing 5 μM C4-CER and 1 × Mg2+-ATP mixture in the presence or absence of 100 ng of SGK1. All reactions were stopped using Laemmli SDS-PAGE sample buffer and subjected to SDS-PAGE, followed by Western blot analysis. SGK1 phosphorylation at Ser422 and Thr256 or PDK1 phosphorylation at Ser241 was immunodetected using an anti-phospho-Ser422 or anti-phospho-Thr256 SGK1 or an anti-phospho-Ser241 PDK1 antibody, respectively.

Rescue of ΔF508-CFTR Trafficking by C4-ceramide
Rescue of ΔF508-CFTR Trafficking by C4-ceramide

**CFTR Immunolocalization**—CFPAC-1 cells were grown in two-well tissue culture slide chambers (Lab-Tek), followed by treatment with 10 μM C4-CER, C4-dCER, or C22-CER or an equivalent amount of vehicle at 37 °C for 20 h. Treated cells were then washed with PBS and fixed in 2% paraformaldehyde (in PBS) for 20 min at room temperature, followed by permeabilization and antigen blocking with 0.2% Triton X-100, 0.2% BSA in PBS for 45 min at room temperature. The nonspecific protein binding sites were blocked by incubation with 1% normal goat serum in PBS, 0.1% Triton X-100, 0.2% BSA for 1 h. Fixed cells were incubated overnight at 4 °C with anti-CFTR C-terminus antibody (1:250) diluted in PBS, 0.1% Triton X-100, 0.2% BSA; washed with PBS, 0.1% Triton X-100 for 10 min; and incubated with Alexa 594-conjugated goat anti-mouse IgG (1:1000; Molecular Probes) in PBS, 0.1% Triton X-100, 0.2% BSA for 1 h at room temperature. After a final wash, slides were mounted in ProLong® gold antifade reagent with DAPI (Molecular Probes) and dried. Images were acquired by confocal microscopy using a Carl Zeiss LSM710 (×63 or ×100 oil immersion objective) equipped with suitable filter packs that allowed the fluorescent molecules to be excited at specific wavelengths. Excitation of Alexa 594 conjugates was carried out with a helium-neon laser, and emission was collected using a 548–644-nm filter set. Signals from the two channels were acquired independently, and the merged images were presented.

**Internalization of Fluorescent NBD-tagged CERs**—CFPAC-1 cells were grown in two-well tissue culture slide chambers (Lab-Tek) and were incubated at 37 °C for 8 h with ETOH/dodecane, 10 μM NBD-C6-CER, or NBD-C18-CER. After washing with PBS, cells were cooled to 4 °C and fixed to trace the intracellular localization of NBD-tagged compounds. Slides were mounted in ProLong® gold antifade reagent with DAPI, and images were acquired by confocal microscopy using a Carl Zeiss LSM710 (×63 or ×100 oil immersion objective) equipped with suitable filter packs that allowed the fluorescent molecules to be excited at specific wavelengths. Signals from the two channels were acquired independently, and the merged images are presented.

**Fluorescence Measurement of Chloride Efflux**—CFPAC-1 cells were grown in 24-well masked tissue culture plates (Wallac, Turku, Finland); treated at 37 °C for 20 h with vehicle or 10 μM C4-CER, C4-dCER, or C22-CER; and then loaded with 10 mM MQAE in culture medium for 8 h at 37 °C. MQAE-loaded cells were then washed with a chloride-rich buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 5 mM glucose, 5 mM HEPES (pH 7.4) and incubated in this buffer for 15 min for chloride concentration equilibration inside and outside of the cells. Chloride transport was induced by changing from a chloride-rich buffer to a chloride-free buffer of similar composition with NO3− as the substituting anion and containing a CAMP-agonist mixture (10 μM forskolin (Calbiochem), 50 μM genistein (Calbiochem), and 500 μM isobutylmethylxanthine (Sigma-Aldrich)) to stimulate CFTR. Over the first few min, the rate of increase in free MQAE fluorescence (arbitrary units) is proportional to the number of chloride channels at the membrane and was monitored over 10 min after buffer exchange using a FLUOstar Optima microplate reader (BMG LabTechnologies). The initial rates ($F_t/F_0$) of chloride transport were calculated from the slopes of the fluorescence curve over the first 180 s, where $F_t$ is the fluorescence at time t, and $F_0$ is the initial fluorescence.

**Measurement of IL-8 Release**—CFPAC-1 cells were treated with vehicle or various concentrations of C4-CER or C4-dCER at 37 °C for 12 h. For experiments using *Pseudomonas aeruginosa* lipopolysaccharide (LPS; Sigma-Aldrich), CFBF cells were initially treated with vehicle or 10 μM C4-CER for 6 h, followed by washing and incubating for an additional 6 h with a fresh dose of vehicle or C4-CER in the presence or absence of 100 ng/ml LPS. After treatment, the culture media were collected for IL-8 measurement, and cells were lysed for determination of the total protein concentration. IL-8 release was determined using a sensitive electrochemiluminescent multiplex ELISA on a Meso Scale Sector 6000 instrument (Meso Scale Discovery, Gaithersburg, MD).

**Culture of Human Primary ΔF508 HBE Cells and Ussing Chamber Assay**—Primary human bronchial epithelial (HBE) cells expressing ΔF508-CFTR were propagated, differentiated, and maintained as described elsewhere (36). ΔF508 HBE cells were cultured on 6.5 mm Costar Transwell filters. C4-CER or C4-dCER (15 μM in DMSO) was then added to the basolateral side of the cell monolayer at 37 °C for 24 h. At the end of drug incubation, filters were mounted in Physiologic Instruments Ussing chambers and allowed to equilibrate for 15 min to permit electrical parameters to stabilize. The basolateral medium consisted of 120 mM NaCl, 25 mM NaHCO3, 3.3 mM KH2PO4, 0.8 mM K2HPO4, 1.2 mM CaCl2, 1.2 mM MgCl2, and 10 mM d-glucose. The apical NaCl solution was replaced with sodium gluconate to achieve a 120 mM transepithelial chloride gradient. The bathing solutions were gassed with 95% O2 and 5% CO2 to maintain a pH of 7.4. Short circuit current ($I_{sc}$) and transepithelial resistance were continuously measured using a Physiologic Instruments VCC-MC8 system and Physiologic Instruments Acquire and Analyze 2.3 data acquisition hardware and software. After the equilibration period, 10 μM amiloride was added to the apical chamber to inhibit epithelial sodium channel-mediated sodium absorption. After 2 min, 10 μM forskolin was added to both the basolateral and apical chambers to activate CFTR-mediated anion excretion. After 2 min, 50 μM genistein was added to the basolateral and apical chambers. After another 2 min, CFTR inhibitor 172 (Sigma-Aldrich) was added to the apical chamber to inhibit CFTR-mediated anion excretion. At these times, currents had achieved steady state. Changes in short circuit current ($D_{sc}$) were calculated from the mean $I_{sc}$ over the 10-s period preceding each drug addition.

**Cell Viability Assay**—A 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyldtetrazolium bromide (MTT) (Sigma-Aldrich) assay was carried out to evaluate the cell viability according to the manufacturer’s instructions. Cells were grown in 24-well plates at a density of 24,000 cells/well and then treated at 37 °C for 20 h with vehicle or various concentrations C4-CER or C4-dCER, followed by washing with PBS. MTT solution was added to each well and incubated at 37 °C for 4 h, after which a formazan-solubilizing solution (DMSO) was added to each well to dissolve the formazan crystals. After 5 min more of incubation, the formazan crystals were solubilized, and the resulting colored solution was quantified by measuring the absorbance at 492 nm.
using a FLUOstar Optima microplate reader. The viability of the treated cells was expressed as a percentage relative to vehicle-treated cells, which was assumed to be 100%.

**Statistical Analysis**—Data are presented as means ± S.E.; n represents the number of different experiments. Statistical significance among means was determined using an unpaired, two-tailed Student’s t test, and p < 0.05 was considered statistically significant.

**RESULTS**

**Short-chain CERs Promote CFTR Maturation and Trafficking to the Plasma Membrane**—Fig. 1A shows extremes of the CER structures tested here from short-chain C2-CER to long-chain C22-CER. When incubated with 10 μM C4-CER for 20 h at 37 °C, pancreatic CFPAC-1 cells express the highest levels of ΔF508-CFTR mature and cell surface band C (Fig. 1B, left). By contrast, short-chain C2-, C6-, and C8-CERs are much less effective, and long-chain C12-, C16-, C18-, and C22-CERs are completely inactive. The ratios of band C/band B (C/B ratio) are summarized as relative densitometry data under the Western blot images. These data show that C2-, C4-, C6-, or C8-CER treatment permits 4-, 22-, 10-, or 6-fold more ΔF508-CFTR, respectively, to accumulate as band C. To determine whether differently sized CERs varied with respect to access to the cytosol, we incubated CFPAC-1 cells with active C6-CER and inactive C18-CER, both tagged with fluorescent NBD. Both molecules appear to have equivalent access to the intracellular space (Fig. 1C). Thus, rescue of ΔF508-CFTR by short-chain CER species is highly dependent on the exact number of carbons in the fatty acyl chain and not due to cell permeability issues encountered by longer acyl chain CERs.

To test whether the process resulting in C4-CER-dependent rescue of ΔF508-CFTR also affects wild-type CFTR, we incubated wild-type CFTR-repaired CFPAC-1 pLJ6 and natural abundance CFTR colon T84 cells with C4-CER or other CERs. Both CFPAC-1 pLJ6 and T84 cells also express higher levels of both mature and cell surface band C when incubated with short-chain CERs but not with their long-chain counterparts (Fig. 1B, middle and right). These data suggest that short-chain CERs target a general trafficking pathway for CFTR, rather than an alternative pathway that is specific for mutant ΔF508-CFTR.

**C4-CER Specifically and Potently Rescues ΔF508-CFTR**—Fig. 2A (left) shows that C4-CER increases the ratio of band C/band B in a dose-dependent manner. The K_{app} is 2.88 ± 0.461 μM. By contrast, C4-CER analog C4-dCER, in which the double bond is reduced, is 20-fold less active (Fig. 2A, right). Time course analysis for the onset of rescue by C4-CER reveals that the threshold is ~4 h after treatment, with maximum rescue at 8–20 h (Fig. 2B). We also analyzed, in parallel, the rescue of ΔF508-CFTR by VX-809, a previously identified small molecule CFTR corrector (37). The effects of equivalent concentrations of these two compounds indicate that C4-CER is approximately twice as effective as VX-809 at promoting cell surface expression of ΔF508-CFTR (Fig. 2C).
We also addressed the question of the stability and function of rescued ΔF508-CFTR at the cell surface. Fig. 2D shows that ΔF508-CFTR can be rescued by low temperature (27 °C) for 48 h. If the culture is shifted to 37 °C in the presence of CHX to preclude further CFTR protein synthesis, the calculated half-life for the 27 °C-rescued band C is 2 h. However, if rescue is activated at 37 °C with 10 μM C4-CER for 20 h, the calculated half-life of C4-CER-rescued band C is ~10 h. Therefore, not only does C4-CER rescue ΔF508-CFTR band C at 37 °C, but it also stabilizes the band C, resulting in a half-life that closely resembles that of wild-type CFTR (38, 39). Immunolocalization studies of ΔF508-CFTR in CFPAC-1 cells also demonstrate that C4-CER markedly elevates plasma membrane-localized CFTR immunofluorescence (Fig. 2E); this plasma membrane-localized fluorescence is not observed when cells are treated with vehicle control, inactive C4-dCER, or inactive C22-CER.

To test whether C4-CER-rescued ΔF508-CFTR supports cAMP-activated chloride conductance, we used MQAE to measure cAMP-activated chloride flux (Fig. 2F) (10). Treatment of CFPAC-1 cells with 10 μM C4-CER for 20 h activates chloride flux by more than 6-fold relative to cells treated with vehicle alone. Consistently, treatment of CFPAC-1 cells with either inactive C4-dCER or inactive C22-CER yields chloride fluxes similar to cells treated with vehicle alone.

Finally, to determine the potential for cytotoxicity by either C4-CER or C4-dCER, we used the MTT method to measure drug effects on metabolic integrity. Neither C4-CER nor C4-dCER treatment is different from the vehicle control after 20 h at a 20 μM concentration (Fig. 2G).

C4-CER Suppresses High Basal IL-8 Release—CF is also known as an inflammatory disorder (40), and IL-8 is overexpressed in cultured CF pancreas and lung epithelial cells (41). We therefore tested whether C4-CER could suppress high basal IL-8 release from CFPAC-1 cells (Fig. 2H). Without added C4-CER, the level of IL-8 release from CFPAC-1 cells is 3.5-fold higher than that from wild-type CFTR-repaired CFPAC-1 plJ6.
cells. Significantly, treatment of the cells with increasing concentrations of C4-CER causes a dose-dependent decrease of IL-8 release. At 20 μM C4-CER, the IL-8 level in CFPAC-1 cells is comparable with that of repaired CFPAC-1 pLJ6 cells. Inactive C4-dCer is less effective in inhibiting IL-8 release. Thus, in addition to rescuing ∆F508-CFTR, C4-CER also suppresses secretion of IL-8.

The Sphingoid Backbone Contributes to C4-CER Rescue Activity—To determine the effect of the sphingoid backbone to C4-CER rescue activity, we synthesized analogues in which the length of the sphingoid backbone was truncated by 4, 8, or 11 carbon atoms (Fig. 3A, left). Western blot analysis shows that shortening the length of the sphingoid backbone proportionately reduces C4-CER rescue activity (Fig. 3A, right). Rescue activities of C4-CER analog 1 (species A1; 4 fewer carbon atoms) and analog 2 (species A2; 8 fewer carbon atoms) are nearly 2- and 4-fold less, respectively, than that of the parent C4-CER. Consistently, C4-CER analog 3 (species A3; 11 fewer carbon atoms) is completely inactive. Comparable sensitivity to truncated sphingoid backbone analogues is also observed for wild-type CFTR in repaired CFPAC-1 pLJ6 cells (Fig. 3A). Based on these findings, a sphingoid backbone length of at least 10 carbons (namely d14[A1] > d10[A2] ≫ d7[A3]) is as critical as the short fatty acyl chain length (namely C4 > C6 > C8 ≫ C2).

We also tested C4-CER analogues in which modifications had been made in the vicinity of the critical double bond (Fig. 3B). We had already shown that reduction of the double bond to C4-dCER resulted in an inactive compound (see Fig. 2A, right). Interestingly, C4-phytoker, which has a hydroxyl group instead of a hydrogen at the carbon 4 position of the sphingoid backbone, is active, with an affinity equivalent to that of C4-CER ($K_{m,app} = 3.18 \pm 0.359 \mu M$). However, neither C4-CER-1-phosphate nor C4-glucosyl-CER efficiently rescues ∆F508-CFTR. Based on these findings, the structure-activity relationship (SAR) for CERs with optimal rescue activity consists of (i) a fatty acyl chain length of 4–6 carbon atoms; (ii) a sphingoid backbone with a minimal length of 10 carbons; and (iii) a double bond between positions 4 and 5 or a hydroxyl group at position 4.

C4-CER, Not Candidate Cellular Metabolites, Rescues ∆F508-CFTR—Fig. 4A illustrates the possibility that C4-CER activity could have been due to the enzymatic conversion to other bioactive sphingolipids. To test this possibility, CFPAC-1 cells were treated with C4-CER in the presence of known chemical inhibitors of the CER metabolic pathway, including d-MAPP, DMS, fumonisin B1, d-PDMP, and NVP-231 (26–30). Western blot analysis shows that C4-CER treatment consistently produces high levels of ∆F508-CFTR band C, even in the presence of the different inhibitors (Fig. 4B). Furthermore, as shown in the same Western blot, the individual inhibitors have no intrinsic rescue activity; nor do they affect levels of band B expression.

A different approach to testing the possibility that C4-CER might be converted into an active sphingolipid species is to directly test the rescue activity of the products of the enzymes affected by the above-tested inhibitors. However, as shown in the Western blot analysis, no rescue activity is detected for SPH, SPH-1-phosphate, C18-CER-1-phosphate, and C18-glucosyl-CER (Fig. 4C). Thus, the rescue activity of C4-CER is not dependent on the conversion of C4-CER to any of the candidate endogenous sphingolipid species or to other downstream metabolic products that might have been generated by the inhibited enzymes.
Rescue of ΔF508-CFTR Trafficking by C4-ceramide

C4-CER Rescues ΔF508-CFTR in Cultured CFBE Cells and in Patient-derived Primary Explants of Human CFBE Cells—Fig. 5A shows that treatment of CFBE cells with increasing concentrations of C4-CER also causes a significant dose-dependent increase in the expression level of both mature and cell surface ΔF508-CFTR band C. The $K_{m, app}$ is $3.39 \pm 0.57 \mu M$. Thus, the affinity of C4-CER rescue activity in CFBE cells is very similar to that found for CFPAC-1 cells. In addition, the substrate specificity for C4-CER-induced rescue in CFBE cells is also similar (Fig. 5B).

We also determined the effect of C4-CER on the stability of ΔF508-CFTR at the cell surface (Fig. 5C). CFBE cells were treated at $37 \degree C$ with C4-CER for 20 h, or incubated at $27 \degree C$ for 48 h, followed by incubation with CHX at $37 \degree C$. Western blot analysis shows that the cell surface half-life of 27 °C-rescued ΔF508-CFTR is $\sim 2$ h. This is similar to the value of the half-life for ΔF508-CFTR band C measured in CFPAC-1 cells following 27 °C rescue. By contrast, the cell surface half-life of C4-CER-rescued [ΔF508] CFTR is $\sim 10$ h, a value very close to that of the parallel experiment in CFPAC-1 cells.

We also tested C4-CER activity in primary cultures of homozygous ΔF508-CFTR HBE cells incubated at the air/liquid interface. In this experiment, samples of C4-CER or inactive C4-dCER were added to the subphase of the Transwell permeable supports for 24 h. Changes in cAMP-activated short circuit current ($I_{sc}$) were determined using an Ussing chamber-based assay. C4-CER treatment increases forskolin/genistein-activated chloride transport by $\sim 2.5$-fold, compared with that of DMSO or inactive C4-dCER (Fig. 5D). Importantly, the increased chloride transport is completely inhibited by a specific CFTR blocker, CFTRinh-172. Taken together, the data suggest that C4-CER is a rescue agent for ΔF508-CFTR that generally affects epithelial cells bearing this common mutation.

Finally, we tested whether C4-CER could suppress basal IL-8 secretion as well as the IL-8 secretion induced by exposure to P. aeruginosa LPS in CFBE cells (Fig. 5E). C4-CER reduces secreted IL-8 levels by $\sim 60\%$. When CFBE cells are treated with LPS, the level of IL-8 secretion nearly doubles. However, C4-CER again lowers the LPS-enhanced IL-8 level by $\sim 60\%$. Thus, the ability of C4-CER to reduce basal or LPS-stimulated IL-8 secretion by CF human airway epithelial cells suggests that C4-CER could be useful as a candidate drug for CF patients because elevated levels of cytokines in the CF airway contribute to lung injury (42).

C4-CER Bypasses PI3K to Rescue ΔF508-CFTR—We previously reported that PI3K is a necessary co-activator of DEX-induced rescue of ΔF508-CFTR (10). We therefore inquired whether PI3K itself might be necessary for C4-CER rescue activity. For this experiment, we transfected CFPAC-1 cells with siRNA directed against two different isoforms of the PI3K catalytic subunit (p110), scrambled control siRNA, or Lamin A/C siRNA duplexes, followed by incubation at $37 \degree C$ for 48 h before 20-h treatment with 10 μM C4-CER (Fig. 6A). Western blot analyses show that siRNA against either α or β PI3K catalytic subunit isoform profoundly reduces endogenous PI3K protein expression. However, the data also show that siRNA knockdown of PI3K catalytic subunit isoforms still permits C4-CER to rescue ΔF508-CFTR.

We also investigated the effects of three well known PI3K inhibitors, wortmannin, LY294002, and PI-103 (31–33), on C4-CER-mediated rescue. Western blot analysis shows that the level of C4-CER-rescued ΔF508-CFTR band C in the presence of wortmannin, LY294002, or PI-103 is comparable with that rescued by C4-CER alone (Fig. 6B). Thus, neither knockdown of endogenous PI3K expression nor inhibition of its activity abolishes C4-CER-mediated rescue, suggesting that it does not require PI3K protein or its kinase activity. To further test this conclusion, we determined whether Akt, a PI3K-activated protein kinase, might be activated in CFPAC-1 cells treated with C4-CER. We compared this outcome with that observed in the same cell line treated with 100 nm DEX, which we had previously reported rescued ΔF508-CFTR in a PI3K-dependent manner (10). Western blot analysis using a phospho-specific antibody to Akt at phosphorylated Ser473 or Thr308 shows that

![Figure 4](image-url)
C4-CER does not increase phosphorylation of Akt at either regulatory site, whereas DEX causes a substantial increase in phosphorylation of Akt at both sites (Fig. 6). Importantly, neither C4-CER nor DEX treatment has any effect on the expression of total Akt protein or β-actin. These three experiments demonstrate that C4-CER rescue activity does not depend on PI3K activation.

**C4-CER-mediated Rescue of ΔF508-CFTR Is Dependent on Both PDK1 and SGK1**—We tested whether C4-CER rescue activity was dependent on PDK1 and SGK1 by down-regulating expression of both PDK1 and SGK1 with specific siRNAs (Fig. 7A). PDK1 siRNA causes an 80% reduction in the PDK1 protein level compared with the scrambled control siRNA. Concomitantly, this level of PDK1 knockdown markedly reduces the expression of both C4-CER-rescued mature and cell surface band C. Likewise, SGK1 siRNA effectively reduces endogenous SGK1 expression to a level of 80% less than that of scrambled control siRNA, and this reduced SGK1 expression substantially reduces C4-CER-rescued mature and cell surface band C. Finally, the same Western blot also shows that, in cells simultaneously transfected with both PDK1 and SGK1 siRNAs, the reduced expression levels of both rescued mature and cell surface band C are further decreased compared with the decrease observed in cells transfected with individual PDK1 or SGK1 siRNAs. By contrast, we saw no changes in the expression of band B or β-actin under these conditions.

We also tested the effects of the PDK1 inhibitor GSK2334470 (34) and the SGK1 inhibitor GSK650394 (35) on C4-CER-mediated rescue (Fig. 7B). Western blot analyses show that 2 μM GSK2334470 or 10 μM GSK650394 suppresses C4-CER rescue activity by 80%. Thus, the levels of C4-CER rescue are equally and proportionally inhibited whether levels of PDK1 and SGK1 proteins are reduced by specific siRNAs or the PDK1 and SGK1 kinase activities are decreased by specific inhibitors. These data suggest that C4-CER bypasses PI3K signaling and directly signals through the PDK1/SGK1 pathway to rescue ΔF508-CFTR.

C4-CER Induces Coincident Phosphorylation and Activation of PDK1 and SGK1—It has been reported that the kinase activity of PDK1 is constitutively active and that PI3K can further activate PDK1 by increasing phosphorylation of the [Raw text continues]
Rescue of ΔF508-CFTR Trafficking by C4-ceramide

FIGURE 6. C4-CER bypasses the PI3K signaling pathway to rescue ΔF508-CFTR in CFPAC-1 cells. A, effect of PI3K knockdown. Cells were transfected with 50 nM scrambled control, PI3K catalytic (p110) subunit isoforms α/β, or Lamin A/C siRNA and then incubated at 37 °C for 48 h prior to treatment with 10 μM C4-CER for 20 h, followed by Western blot analysis for ΔF508-CFTR expression. B, effects of PI3K inhibitors. Cells were treated at 37 °C for 20 h with vehicle control or 10 μM C4-CER in the presence or absence of the indicated PI3K inhibitors, followed by Western blot analysis for ΔF508-CFTR expression. C, effect of C4-CER on phosphorylation of Akt. Cells were treated at 37 °C for 20 h with vehicle control, 10 μM C4-CER, or 100 nM DEX, followed by Western blot analysis for Akt phosphorylation at Thr308 or Ser473. Total Akt and β-actin were used as phosphorylation and loading controls, respectively. For all experiments, a representative Western blot of three independent experiments is shown.

protein at Ser241 (43, 44). We therefore tested whether C4-CER induced phosphorylation of PDK1 at Ser241. Fig. 7C (top) shows that increasing concentrations of C4-CER cause a dose-dependent increase in PDK1 phosphorylation at Ser241 (namely PDK1[Ser(P)241]). By contrast, inactive C4-dCER or C22-CER (Fig. 8A) shows that increasing concentrations of C4-CER cause dose-dependent increases in SGK1[Ser(P)422] phosphorylation of recombinant SGK1. By contrast, as shown in the same Western blot, omission of Mg2+-ATP from the kinase reaction or the addition of inactive C4-dCER results in very low levels of SGK1[Ser(P)422] phosphorylation.

Interestingly, we find that in an in vitro kinase reaction, incubation of PDK1 with C4-CER alone does not enhance Ser241 autophosphorylation of PDK1, but it does when SGK1 is added (Fig. 8B). The data therefore indicate that the effect of C4-CER in activation of the PDK1/SGK1 signaling is initially on SGK1 rather than PDK1. At present, how C4-CER mediates the autophosphorylation event of SGK1 is unclear. Because AGC kinases possess lipid binding domains, one possibility is that C4-CER first binds to SGK1 and induces a conformational change in SGK1, subsequently allowing the enzyme to autophosphorylate at Ser422, and second, brings PDK1 in close proximity to SGK1. This enables PDK1 to directly interact with the phosphorylated Ser422 of SGK1, which subsequently leads to an increase in Ser241 autophosphorylation and thus activity of PDK1. The PDK1 activation following the interaction of PDK1 with the phosphorylated Ser422 of SGK1 has been documented in the literature (48, 49).

Additional Western blot analyses show that SGK1[Thr(P)256, Ser(P)422] and PDK1[Ser(P)241] phosphorylation is markedly enhanced in the kinase reaction containing C4-CER but not inactive C4-dCER or C22-CER (Fig. 8C). Presumably, at the cellular level, C4-CER-mediated activation of the PDK1/SGK1 signaling can be achieved through this mechanism, independent of endogenous long-chain CERs and of PI3K-dependent mTORC2 activity. Indeed, inhibition of PI3K signaling (see Fig. 4A) or knockdown of Rictor, a subunit of mTORC2 (Fig. 8D), still allows C4-CER to rescue ΔF508-CFTR.

C4-CER Blocks Formation of the Ned4-2–ΔF508-CFTR Complex—Ned4-2 that has been phosphorylated by SGK1 cannot bind and ubiquitinate ΔF508-CFTR, thereby permitting functional rescue of ΔF508-CFTR (10). We therefore tested whether C4-CER-dependent activation of PDK1/SGK1 signaling would also block binding of Ned4-2 to ΔF508-CFTR. Fig. 9A shows that exposure of CFPAC-1 cells to C4-CER reduces (third and fourth panels). By contrast, C4-dCER is relatively inactive in both cases. No changes in the levels of expression of total Ned4-2 or NDRG1 are detectable under any of the experimental conditions.

C4-CER Induces Autophosphorylation of PDK1 and SGK1—Phosphorylation of SGK1 at Ser422, which is the initial step for its subsequent Thr256 phosphorylation by PDK1, is controlled by mTOR complex 2 (mTORC2) in a PI3K-dependent manner (14). However, the above data show that C4-CER rescues ΔF508-CFTR via the PDK1/SGK1 pathway in a PI3K-independent manner, suggesting that SGK1 utilizes a different mechanism to regulate Ser422 phosphorylation. Because PDK1 and SGK1 belong to the same AGC (protein kinase A, G, and C) kinase family and PDK1 has been shown to possess intrinsic autophosphorylation ability (45), it is possible that SGK1 auto-phosphorylates. We therefore used in vitro kinase assays and purified recombinant SGK1 to test whether C4-CER could induce autophosphorylation of SGK1 at Ser422. Fig. 8A shows that increasing concentrations of C4-CER cause dose-dependent increases in SGK1[Ser(P)422] phosphorylation of recombinant SGK1. By contrast, as shown in the same Western blot, omission of Mg2+-ATP from the kinase reaction or the addition of inactive C4-dCER results in very low levels of SGK1[Ser(P)422] phosphorylation.
the binding of Nedd4-2 to ΔF508-CFTR while increasing the level of Nedd4-2[Ser(P)]468. Consistently, the level of rescued band C is also substantially elevated. Fig. 9A also shows that when the incubation includes either PDK1 inhibitor GSK2334470 or SGK1 inhibitor GSK650394, Nedd4-2 is not phosphorylated. Consequently, Nedd4-2 binds to ΔF508-CFTR, and there is no elevation in mature band C. Thus, C4-CER appears to rescue ΔF508-CFTR by activating PDK1/SGK1 signaling, which in turn phosphorylates Nedd4-2. This prevents binding of Nedd4-2 to ΔF508-CFTR, permitting the mutant protein to traffic to the plasma membrane.

**SGK1 Interacts with CFTR and Induces CFTR Phosphorylation**—SGK1, but not its kinase-dead form, has been reported to activate recombinant wild-type CFTR chloride currents in Xenopus oocytes by a mechanism involving an increase in the number of chloride channels in the membrane (50). The above data show that C4-CER increases the level of both mature and cell surface band C in wild-type CFTR-bearing cells (see Fig. 1B). We therefore conjectured that rescue of ΔF508-CFTR by C4-CER might include SGK1-mediated phosphorylation of the mutant protein. Fig. 9B shows that, in the presence of C4-CER, phosphorylation of both wild-type and ΔF508-CFTR immunoprecipitates can be detected with an antibody against a common Ser(P)/Thr(P) epitope. Consistently, SGK1 co-immunoprecipitates with both CFTR forms. However, phosphorylated SGK1[Thr(P)256] is only detected in CFTR immunoprecipitates if C4-CER is also included in the cell treatment. These data indicate that SGK1 physically interacts with mutant and wild-type CFTR, and, following C4-CER treatment, bound SGK1 is phosphorylated and converted into an activated form, SGK1[Ser(P)422, Thr(P)256], which catalyzes phosphorylation of CFTR.

We also tested whether active recombinant PDK1 could activate SGK1, resulting in the phosphorylation of immunoprecipitated CFTR (Fig. 9C). The *in vitro* kinase reaction mixture consists of active PDK1, mutant S422D-SGK1, and substrate CFTR that was immunoprecipitated from either untreated CFPAC-1 or repaired CFPAC-1 pLJ6 cells. The S422D mutation of SGK1, a mimic in which its hydrophobic motif phosphorylation site is changed to an acidic residue, allows the protein to be more readily phosphorylated at Thr256 and hence activated by PDK1 (48, 49). Western blot analysis shows that active PDK1 drives phosphorylation of mutant SGK1, resulting in Thr(P)/Ser(P) phosphorylation of either ΔF508-CFTR band B or wild-type CFTR band B and band C. However, if active PDK1 is omitted from the reaction, very little Thr(P)/Ser(P) CFTR labeling occurs. Consistently, without PDK1, virtually no mutant SGK1 is phosphorylated at Thr256. The phosphorylation data shown here does not exclude the contribution of endogenous SGK1.
that is co-immunoprecipitated with mutant and wild-type CFTR and is activated by PDK1.

The functional meaning of the above result is that, in the presence of C4-CER, mutant CFTR evades ubiquitination by Nedd4-2 by carrying its own SGK1[Ser(P)422, Thr(P)256]. Indeed, when the ΔF508-CFTR immunoprecipitates from control cell lysates are subjected to Western blot analysis with an anti-ubiquitin antibody, a distributed high molecular mass signal ranging from 140 to 200 kDa is specifically observed (Fig. 9A). By contrast, ΔF508-CFTR immunoprecipitates from C4-CER-treated cell lysates display a substantial reduction in the distributed ubiquitinated signal. Importantly, this reduction is accompanied by the appearance of band C in the mutant CFTR immunoprecipitates.

**DISCUSSION**

In this paper, we show that C4-CER functionally rescues ΔF508-CFTR in cultured CF pancreas and lung cells as well as in primary cultures of homozygous ΔF508-CFTR human bronchial epithelial cells. The functions supported by C4-CER include activation of cAMP-activated chloride channel conductance, increased stability in the membrane-bound state, and suppression of IL-8 secretion. C4-CER-mediated rescue of ΔF508-CFTR depends on the integrity of the PDK1/SGK1 pathway but is independent of the PI3K/mTOR pathway or glucocorticoids. We also show that C4-CER can replace the PI3K/mTORC2 pathway to directly induce SGK1 to autophosphorylate at Ser422, an initial step leading to activation of PDK1 and of SGK1 by PDK1. As summarized in Fig. 10, the rescue mechanism is initiated by binding of C4-CER to SGK1 that leads to autophosphorylation of the protein at Ser422. SGK1[Ser(P)422] and C4-CER coincidently bind PDK1 and permit PDK1 to autophosphorylate at Ser241. Then PDK1[Ser(P)241] phosphorylates SGK1[Ser(P)422] at Thr256 to generate fully activated SGK1[Ser(P)422, Thr(P)256]. SGK1[Ser(P)422, Thr(P)256] now phosphorylates Nedd4-2 at Ser668 to form Nedd4-2[Ser(P)668]. This phosphorylation step prevents binding and ubiquitination of CFTR by Nedd4-2. From this perspective, ΔF508-CFTR appears to carry SGK1 as a molecular “bodyguard,” which can be activated by C4-CER, through PDK1 activation, to directly phosphorylate and protect CFTR from Nedd4-2. The ΔF508-CFTR trafficking defect is thus corrected, and the newly rescued, functional protein is free to move to the apical plasma membrane.

Recent reports suggest that CF may actually be associated with a defect in sphingolipid metabolism (51–53). However, these findings are controversial. For example, Radzioch’s group (51, 52) has reported that diminished levels of endogenous long-chain CERS are found in CF-relevant organs, both in patients with CF and in CFTR knock-out (KO) mice. By contrast, Gulbins’ group (53) has reported that, both in patients with CF and in CFTR KO mice, increased levels of endogenous long-chain CERS are found in CF-relevant organs. Parenthetically, these studies have not mentioned whether increased or decreased levels of endogenous long-chain CERS have any effects on rescuing ΔF508-CFTR. However, we find that by using the well established ETOH/dodecane delivery vehicle for long-chain CERS (17–25), directly adding these CERS with varying acyl chain lengths as well as other bioactive sphingolipids (i.e. SPH, SPH-1-P, C24-CER-1-P, and C18-Glc-CER) to CF cells fails to rescue ΔF508-CFTR. Instead, we find that only treating CF cells with synthetic short-chain CERS can promote rescue of ΔF508-CFTR (see Figs. 1 and 4). Thus, natural sphingolipids are not rescue agents.

From the detailed analysis of synthetic CER analogues with similar cell permeabilities, C4-CER emerges as the most potent “hit.” The SAR for these CERS can now be discerned. First of all, the acyl group is optimally 4 carbons. This is defined by a virtually bell-shaped activity series: C4 > C6 > C8 >> C2 (see Fig. 1B). The second defining structural requirement is a limitation on the sphingoid backbone, which must be at least 10 carbons in length (see Fig. 3A). A third defining structural requirement is that the double bond in the sphingoid backbone at the C4-C5 positions cannot be reduced to the dihydro form (namely C4-dCER; see Fig. 2). However, hydroxylation of the dihydro form at the C4 position is permitted (namely C4-phytoCER; see Fig. 3B), with virtually full retention of activity of C4-CER. Note that phytoCER is the predominant CER in plants and yeast (54). Fourth, substitutions of the C1 position by either glucosyl or phosphate moieties destroy rescue activity, indicating that the
optimal substitution at the C1 position is at least only a hydroxyl group (see Fig. 3B). Based on this SAR, rescue activities of short-chain CERs specifically depend on the acyl chain length or the integrity of exogenously added CERs rather than on CER metabolism or uptake. Such acyl chain length specificity as shown here is relatively similar to those reported by other groups on other CER-mediated biological effects (23–25). It is possible that CER with a proper acyl chain length has the most effective molecular conformation to facilitate the molecule’s interaction with and thus activation of its targets. Consistently, we find that C4-CER, but not C22-CER, effectively induces in vitro phosphorylation and thus activation of both SGK1 and PDK1 (see Fig. 8C) and drives the PDK1/SGK1 rescue pathway for ΔF508-CFTR.

The close relationship between SGK1 and CFTR has been documented and is further substantiated in our present work. Sato et al. (50) showed that the interaction between CFTR and SGK1 depends on the C-terminal PDZ binding domain in SGK1 interacting with the PDZ domain of CFTR. Based on co-immunoprecipitation experiments, the work described in the present paper supports the concept that SGK1 binds to both
Rescue of ΔF508-CFTR Trafficking by C4-ceramide

wild type and ΔF508-CFTR. Furthermore, the importance of active SGK1 kinase for the addition of CFTR to the plasma membrane in Xenopus oocytes (50) is consistent with our observation that PDK1-activated S422D-SGK1 phosphorylates both wild-type and mutant CFTR. We cannot exclude the possibility that the direct phosphorylation of CFTR by C4-CER-activated SGK1 plays a role in the rescue process in cultured cells. For instance, the introduction of the suppressor mutation V510D to ΔF508-CFTR mediates rescue of ΔF508-CFTR and increases the half-life of the membrane-bound ΔF508-CFTR (39). Presumably, the addition of a negative charged phospho group to the mutant protein by SGK1 might also overcome the negative effects of the ΔF508 mutation in a similar manner. The consensus SGK1 phosphorylation sequence is RXRX(S/T)φ (where φ is a hydrophobic residue) (13). In prospect, CFTR does have two potential consensus sequences for phosphorylation by SGK1 in the NBD1 domain. These are DEYRRYRS519V and GGQRARIS557L. However, the identification of the actual phosphorylation sites on CFTR and their function remains an unsolved problem, which we will address in the future.

Alternatively, SGK1 can regulate membrane protein trafficking through the SGK1-PIKfyve (phosphatidylinositol-3-phosphate-5-kinase) pathway. PIKfyve has been shown to be involved in the regulation of endosome-to-Golgi retrograde transport (55) and also in regulating membrane protein trafficking, including CFTR (56). One study shows that PIKfyve is phosphorylated by SGK1, leading to activation of the kinase with subsequent production of phosphatidylinositol 3,5-biphosphate, which then activates the Rab11-dependent exocytosis of KCNQ1/KCNE1 (57). With regard to mutant CFTR, ΔF508-CFTR can be efficiently recycled when the Rab11-dependent recycling machinery is up-regulated (58). Further studies will be necessary to determine whether C4-CER-mediated rescue of ΔF508-CFTR recycling takes advantage of this pathway. Collectively, C4-Cer rescues and stabilizes ΔF508-CFTR through the PDK1/SK1 pathway by (i) inhibiting the formation of ΔF508-CFTR-Nettd4-2 complex, (ii) possibly stabilizing interdomain interactions following direct phosphorylation at these potential SGK1 phosphorylation sites, and (iii) possibly promoting Rab11-mediated recycling machinery via SGK1-activated PIKfyve.

It is possible that C4-CER might be useful as a candidate CF therapeutic compound. At the cellular level, we have been unable to demonstrate toxicity using the classical MTT viability assay. From a pharmacologic point of view, C4-CER rescues CFTR activity in multiple CF cell lines and in primary HBE cells cultivated from human CF patient lung explants. This result is consistent with a general chemical effect on ΔF508-CFTR in the context of human cells. Finally, the suppressive effects of C4-CER on elevated IL-8 secretion suggests that the rescue of ΔF508-CFTR has consequences for many of the defects associated with CF, including the massively proinflammatory phenotype. We therefore cannot exclude the possibility that C4-CER may have some general anti-inflammatory effects beyond those due solely to the rescue of ΔF508-CFTR.

REFERENCES

1. Riordan, J. R. (1999) Cystic fibrosis as a disease of misprocessing of the cystic fibrosis transmembrane conductance regulator protein. Am. J. Hum. Genet. 64, 1499–1504
2. Anderson, M. P., Sheppard, D. N., Berger, H. A., and Welsh, M. J. (1992) Chloride channel in the apical membrane of normal and cystic fibrosis airway and intestinal epithelia. Am. J. Physiol. 263, L1–L14
3. Srivastava, M., Eidelman, O., Zhang, J., Paweletz, C., Caohuy, H., Yang, Q., Jacobson, K. A., Heldman, E., Huang, W., Jozwik, C., Pollard, B. S., and Pollard, H. B. (2004) Digitoxin mimics gene therapy with CFTR and suppresses hypersecretion of IL-8 from cystic fibrosis lung epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 101, 7693–7698
4. Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O’Riordan, C. R., and Smith, A. E. (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 63, 827–834
5. Eidelman, O., Guay-Broder, C., van Galen, P. J., Jacobson, J. F., Fox, C., Turner, R. J., Cabantchik, Z. I., and Pollard, H. B. (1992) A1 adenosine-receptor antagonists activate chloride efflux from cystic fibrosis cells. Proc. Natl. Acad. Sci. U.S.A. 89, 5562–5566
6. Vij, N., Fang, S., and Zetlin, P. L. (2006) Selective inhibition of endoplasmic reticulum-associated degradation rescues ΔF508-cystic fibrosis transmembrane regulator and suppresses interleukin-8 levels: therapeutic implications. J. Biol. Chem. 281, 17359–17378
7. Dechecchi, M. C., Nicolis, E., Bezzerr, V., Vella, A., Colombatti, M., As sael, B. M., Mettey, Y., Borgatti, M., Mancini, I., Gambrai, B., Becq, F., and Cabrini, G. (2007) MPB-07 reduces the inflammatory response to Pseudomonas aeruginosa in cystic fibrosis bronchial cells. Am. J. Respir. Cell Mol. Biol. 36, 615–624
8. Cholon, D. M., O’Neal, W. K., Randell, S. H., Riordan, J. R., and Gentasch, M. (2010) Modulation of endocytic trafficking and apical stability of CFTR in primary human airway epithelial cultures. Am. J. Physiol. Lung Cell Mol. Physiol. 298, L304–L314
9. Pedemonte, N., Zegarra-Moran, O., and Galietta, L. J. (2011) High-throughput screening of libraries of compounds to identify CFTR modulators. Methods Mol. Biol. 741, 13–21
10. Caohuy, H., Jozwik, C., and Pollard, H. B. (2009) Rescue of ΔF508-CFTR by the SGK1/Nedd4-2 signaling pathway. J. Biol. Chem. 284, 25241–25253
11. Bhattacharrya, S., Balakathiresan, N. S., Dalgard, C., Gutt, U., Armitstead, D., Jozwik, C., Srivastava, M., Pollard, H. B., and Biswas, R. (2011) Elevated miR-15 promotes inflammation in cystic fibrosis by driving hyperexpression of IL-8. J. Biol. Chem. 286, 11604–11615
12. Sheppard, K., Kinross, K. M., Solomon, B., Pearson, R. B., and Phillips, W. A. (2012) Targeting PI3 kinase/AKT/mTOR signaling in cancer. Crit. Rev. Oncog. 17, 69–95
13. Kobayashi, T., and Cohen, P. (1999) Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositol-3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. Biochem. J. 339, 319–328
14. Garcia-Martinez, J. M., and Alessi, D. R. (2008) mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1) Biochem. J. 416, 375–385
15. Casamayor, A., Torrance, P. D., Kobayashi, T., Thorner, J., and Alessi, D. R. (1999) Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. Curr. Biol. 9, 186–197
16. Friant, S., Lombardi, R., Schmelze, T., Hallen, M. N., and Riezman, H. (2001) Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. EMBO J. 20, 6783–6792
17. Li, L., Zhang, G., Uematsu, S., Akahori, Y., and Hirabayashi, Y. (1995) Induction of apoptotic DNA fragmentation and cell death by natural ceramide. FEBS Lett. 358, 211–214
18. Gomez-Muñoz, A., Frago, L. M., Alvarez, L., and Varela-Nieto, I. (1997) Stimulation of DNA synthesis by natural ceramide 1-phosphate. Biochem. J. 325, 435–440
19. Paris, F., Grassmé, H., Cremesti, A., Zager, J., Fong, Y., Haimovitz-Fried-
man, A., Fuk, Z., Gulbins, E., and Kolesnick, R. (2001) Natural ceramide reverses Fas resistance of acid sphingomyelinase(−/−) hepatocytes. J. Biol. Chem. 276, 8297–8305

20. Ardail, D., Popa, I., Bodencen, J., Famy, C., Louisop, P., and Portoukalion, J. (2002) Subcellular distribution and metabolic fate of exogenous ceramides taken up by HL-60 cells. Biochim. Biophys. Acta 1583, 305–310

21. Pettus, B. J., Bielawska, A., Subramanian, P., Wijesinghe, D. S., Maceyka, M., Leslie, C. C., Evans, J. H., Freiberg, J., Roddy, P., Hannun, Y. A., and Chalfant, C. E. (2009) Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. J. Biol. Chem. 279, 11320–11326

22. Pettus, B. J., Kitatani, K., Chalfant, C. E., Taha, T. A., Kawamori, T., Bielawska, A., Greenberg, M. S., Perry, D., Jayadev, S., Shayman, J. A., Babenko, N., Hassouneh, L., Budvytiene, M., Liesiene, J., and Geilen, C. (2010) Natural C18:0-ceramide induces cellular sphingolipid accumulation and apoptosis. WebmedCentral APOPTOSIS 1, WMC001100

23. Yatomi, Y., Ruan, F., Megidish, T., Toyokuni, T., Hakomori, S., and Igarashi, Y. (1996) N6-Dimethylphosphine inhibition of sphingosine kinase and sphingosine 1-phosphate activity in human platelets. Biochemistry 35, 626–633

24. Merrill, A. H., Jr., van Echten, G., Wang, E., and Sandhoff, K. (1993) Fusonsin B1 inhibits sphingosine (sphinganine) N-acetyltransferase and de novo sphingolipid biosynthesis in cultured neurons in situ. J. Biol. Chem. 268, 27299–27306

25. Li, L., Ståhlman, M., Ruberg, M., Häversen, L., Fogelstrand, P., Andersson, L., Levin, M., and Borén, J. (2011) ARF6 regulates neuron differentiation through glucosyl-ceramide synthase. PLoS One 6, e260118

26. Graf, C., Rovina, P., and Bornancin, F. (2009) A secondary assay for ceramide kinase inhibitors based on cell growth inhibition by short-chain ceramides. Anal. Biochem. 384, 166–169

27. Arcaro, A., and Wymann, M. P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem. J. 296, 297–301

28. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase: 2-(4-morpholinyl)-8-phenyltrisphosphate in neutrophil responses. J. Biol. Chem. 270, 12646–12654

29. Sato, J. D., Chapline, M. C., Thibodeau, R., Frizzell, R. A., and Stanton, B. A. (2001) Regulation of human cystic fibrosis transmembrane conductance regulator by the investigational drug VX-809. Proc. Natl. Acad. Sci. U.S.A. 108, 18843–18848

30. VanGoor, F., Hadida, S., Grootenhuis, P. D., Burton, B., Stack, J. H., Straley, K. S., Decker, C. J., Miller, M., McCartney, J., Olson, E. R., Wine, J. J., Frizzell, R. A., Ashlock, M., and Negulescu, P. A. (2011) Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. Biochim. Biophys. Acta 1814, 10843–10848

31. Gulbins, E., Teichgraber, V., and Frizzell, R. A. (2000) A secondary assay for ceramide-1-phosphate and ceramide-1-phosphate. Mol. Pharmacol. 68, 330–335

32. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyltrisphosphate in neutrophil responses. J. Biol. Chem. 270, 12646–12654

33. Li, L., Ståhlman, M., Ruberg, M., Häversen, L., Fogelstrand, P., Andersson, L., Levin, M., and Borén, J. (2011) ARF6 regulates neuron differentiation through glucosyl-ceramide synthase. PLoS One 6, e260118

34. Graf, C., Rovina, P., and Bornancin, F. (2009) A secondary assay for ceramide kinase inhibitors based on cell growth inhibition by short-chain ceramides. Anal. Biochem. 384, 166–169

35. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase: 2-(4-morpholinyl)-8-phenyltrisphosphate in neutrophil responses. Biochem. J. 296, 297–301

36. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase: 2-(4-morpholinyl)-8-phenyltrisphosphate in neutrophil responses. Biochem. J. 296, 297–301

37. Sherk, A. B., Frigo, D. E., Schnackenberg, C. G., Bray, J. D., Laping, N. I., Trizna, W., Hammond, M., Patterson, L., Thompson, S. K., Kazmin, D., Norris, J. D., and McDonnell, D. P. (2008) Development of a small-molecule serum- and glucocorticoid-regulated kinase-1 antagonist and its evaluation as a prostate cancer therapeutic. Cancer Res. 68, 7475–7483

38. Holleran, J. P., Glover, M. L., Peters, K. W., Bertrand, C. A., Watkins, S. C., Jarvis, J. W., and Frizzell, R. A. (2012) Pharmacological rescue of the mutant cystic fibrosis transmembrane conductance regulator (CFTR) detected by use of a novel fluorescence protocol. Mol. Med. 18, 685–696

39. Guo, F., Hadida, S., Grootenhuis, P. D., Burton, B., Stack, J. H., Straley, K. S., Decker, C. J., Miller, M., McCartney, J., Olson, E. R., Wine, J. J., Frizzell, R. A., Ashlock, M., and Negulescu, P. A. (2011) Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. Proc. Natl. Acad. Sci. U.S.A. 108, 18843–18848

40. Gulbins, E., Teichgraber, V., and Frizzell, R. A. (2000) A secondary assay for ceramide-1-phosphate and ceramide-1-phosphate. Mol. Pharmacol. 68, 330–335

41. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyltrisphosphate in neutrophil responses. J. Biol. Chem. 270, 12646–12654

42. Sherk, A. B., Frigo, D. E., Schnackenberg, C. G., Bray, J. D., Laping, N. I., Trizna, W., Hammond, M., Patterson, L., Thompson, S. K., Kazmin, D., Norris, J. D., and McDonnell, D. P. (2008) Development of a small-molecule serum- and glucocorticoid-regulated kinase-1 antagonist and its evaluation as a prostate cancer therapeutic. Cancer Res. 68, 7475–7483

43. Holleran, J. P., Glover, M. L., Peters, K. W., Bertrand, C. A., Watkins, S. C., Jarvis, J. W., and Frizzell, R. A. (2012) Pharmacological rescue of the mutant cystic fibrosis transmembrane conductance regulator (CFTR) detected by use of a novel fluorescence protocol. Mol. Med. 18, 685–696
Rescue of ΔF508-CFTR Trafficking by C4-ceramide

J. G., Stenmark, H., and Cullen, P. J. (2006) The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. *J. Cell Sci.* **119**, 3944–3957

56. Pakladok, T., Almilaji, A., Munoz, C., Alesutan, I., and Lang, F. (2013) PIKfyve sensitivity of hERG channels. *Cell Physiol. Biochem.* **31**, 785–794

57. Seebohm, G., Strutz-Seebohm, N., Birkin, R., Dell, G., Bucci, C., Spinosa, M. R., Baltaev, R., Mack, A. F., Korniychuk, G., Choudhury, A., Marks, D., Pagano, R. E., Attali, B., Pfeuffer, A., Kass, R. S., Sanguinetti, M. C., Tavare, J. M., and Lang, F. (2007) Regulation of endocytic recycling of KCNQ1/KCNE1 potassium channels. *Circ. Res.* **100**, 686–692

58. Gentzsch, M., Chang, X. B., Cui, L., Wu, Y., Ozols, V. V., Choudhury, A., Pagano, R. E., and Riordan, J. R. (2004) Endocytic trafficking routes of wild type and ΔF508 cystic fibrosis transmembrane conductance regulator. *Mol. Biol. Cell* **15**, 2684–2696