Rescue of ΔF508-CFTR by the SGK1/Nedd4-2 Signaling Pathway*

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The most common mutation in cystic fibrosis (CF) is ΔF508, which is associated with failure of the mutant cystic fibrosis transmembrane conductance regulator (CFTR) to traffic to the plasma membrane. By a still unknown mechanism, the loss of correctly trafficked ΔF508-CFTR results in an excess of the epithelial sodium channel (ENaC) on the apical plasma membrane. ENaC trafficking is known to be regulated by a signaling pathway involving the glucocorticoid receptor, the serum- and glucocorticoid-regulated kinase SGK1, and the ubiquitin E3 ligase Nedd4-2. We show here that dexamethasone rescues functional expression of ΔF508-CFTR. The half-life of ΔF508-CFTR is also dramatically enhanced. Dexamethasone-activated ΔF508-CFTR rescue is blocked either by the glucocorticoid receptor antagonist RU38486 or by the phosphatidylinositol 3-kinase inhibitor LY294002. Co-immunoprecipitation studies indicate that Nedd4-2 binds to both wild-type- and ΔF508-CFTR. These complexes are inhibited by dexamethasone treatment, and CFTR ubiquitination is concomitantly decreased. We further show that knockdown of Nedd4-2 by small interfering RNA also corrects ΔF508-CFTR trafficking. Conversely, knockdown of SGK1 by small interfering RNA completely blocks dexamethasone-activated ΔF508-CFTR rescue. These data suggest that the SGK1/Nedd4-2 signaling pathway regulates both CFTR and ENaC trafficking in CF epithelial cells.

Cystic fibrosis (CF)² is the most common life-limiting genetic disease in the United States and is due to mutations in the CFTR gene. The most common mutation, ΔF508-CFTR, results in a failure of the mutant protein to traffic properly to the apical plasma membrane of epithelial cells in the lung and other organs (1, 2). By a still unknown mechanism, the loss of correctly trafficked ΔF508-CFTR results in an excess of the epithelial sodium channel (ENaC) on the apical plasma membrane (3–5). In the CF lung, such high levels of ENaC activity are believed to cause dehydration of the airway, and the consequent proinflammatory condition that characterizes CF lung pathophysiology. Similar proinflammatory pathophysiology has been reported to characterize the lung of transgenic mice which overexpress β-ENaC (6). Operationally, it seems that when membrane-localized CFTR decreases in CF, ENaC activity at the plasma membrane increases; CF-related morbidity and mortality follow.

In the case of ENaC trafficking, the process is known to be regulated by a glucocorticoid receptor/SGK1 signaling pathway affecting phosphorylation of the ubiquitin ligase E3 protein Nedd4-2 (7, 8). Fig. 1 illustrates how surface expression of ENaC is controlled by the serum- and glucocorticoid-inducible kinase SGK1, the upstream signal, and the ubiquitin E3 ligase Nedd4-2, the downstream signal. Under default conditions, Nedd4-2 suppresses ENaC surface expression by binding to ENaC via the interaction between the PXXY motifs of ENaC and WW domains on Nedd4-2. Nedd4-2 then catalyzes the ubiquitination of bound ENaC. This step targets ENaC for proteasomal degradation (9, 10). However, when Nedd4-2 is phosphorylated by SGK1, the default interaction between Nedd4-2 and ENaC is reduced, and ENaC is maintained at the plasma membrane (7, 8). The requirement for Nedd4-2 for destruction of ENaC is supported by the recent observation that siRNA against Nedd4-2 is sufficient to permit ENaC to be expressed at the plasma membrane (10). Importantly, both glucocorticoid receptor (GR) and phosphoinositide-3-kinase (PI 3-kinase) signaling pathways must be present for high levels of Na⁺ transport to occur. For example, treatment with the GR antagonist RU38486 (11–13) or the PI 3-kinase inhibitor LY294002 (14–16) results in a complete loss of glucocorticoid-activated ENaC activity.

The placement of the parenthetical (CFTR) in the SGK1/Nedd4-2 signaling pathway (Fig. 1) serves to underscore our hypothesis that CFTR itself could play an interactive or parallel role in the SGK1/Nedd4-2/ENaC-trafficking mechanism. This hypothesis seems reasonable because the regulatory effects of SGK1 and Nedd4-2 are not limited to trafficking of ENaC but also regulate several other epithelial channels and transporters (17, 18). Additionally, co-expression studies in Xenopus oocytes (19, 20) have shown that SGK1 appears to greatly enhance the functional activity of CFTR.

In this report we have shown that activation of the SGK1 signaling pathway by the glucocorticoid dexamethasone results in the rescue of ΔF508-CFTR. The half-life of ΔF508-
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CFTR, once it reaches the plasma membrane, is also dramatically enhanced. Consistently, glucocorticoid-activated ΔF508-CFTR rescue is blocked by the GR antagonist RU38486 and by the PI 3-kinase inhibitor LY294002 as well as by knockdown of endogenous SGK1 by siRNA. We have further shown that at the downstream end of the SGK1/Nedd4-2 signaling pathway, knockdown of Nedd4-2 by siRNA also results in ΔF508-CFTR rescue. Finally, co-immunoprecipitation studies indicated that Nedd4-2 binds to both WT- and ΔF508-CFTR and that treatment with either glucocorticoid or Nedd4-2 siRNA reduces formation of Nedd4-2-CFTR complexes as well as ubiquitination of ΔF508-CFTR. Consistently, chloride transport is well correlated with the level of plasma membrane expression of ΔF508-CFTR protein. These data suggest that the glucocorticoid-receptor-dependent SGK1/Nedd4-2 signaling pathway regulates both CFTR and ENaC trafficking in CF epithelial cells. We interpret these results to indicate that drugs affecting the SGK1/Nedd4-2 signaling pathway may be promising targets for cystic fibrosis therapeutic development.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Drug Treatments**—CFPAC-1 cells, derived from a pancreatic duct adenocarcinoma from a CF patient homozygous for ΔF508-CFTR, have the ion transport properties of CF-affected epithelia (21). Repaired CFPAC-1 pLJ6 cells, created by transfecting CFPAC-1 cells with the pLJ retrovirus carrying wild-type CFTR (22), were kindly provided by Dr. Raymond A. Frizzell. Cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) in a 5% CO₂ environment. The glucocorticoid dexamethasone (DEX; Sigma-Aldrich) was prepared fresh in ethanol, and the final ethanol concentration used was 1% or less. Cells were grown at 37 °C in 10-cm² culture dishes until 70% confluence and then treated with or without various concentrations of DEX for 48 h, with a change of medium containing DEX after the first 24-h incubation. For the pretreatment with chemical inhibitors, cells were incubated at 37 °C with either 1 μM RU38486 (Sigma-Aldrich) or 1–2 μM LY294002 or LY303511 (Calbiochem) for 24 h before and during a 48-h treatment with 50 nM DEX.

For the half-life measurement of membrane-associated CFTR, repaired and CF cells were treated with or without 50 nM DEX in complete DMEM medium for 48 h (pulse). Cells were then washed with DEX-free medium and incubated at 37 °C in DMEM containing 20 μg/ml of cycloheximide (Sigma-Aldrich) for the indicated periods of time (chase).

**Cell-surface Biotinylation Assay**—Cell-surface proteins were biotinylated using EZ-Link sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithio-propionate (NHS-SS-biotin, 1 mg/ml in PBS, pH 8.2; Pierce (23)). Drug-treated or siRNA-transfected cells were rapidly cooled to 4 °C, washed in cold PBS, pH 8.2, supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂, and then incubated with 3 ml of PBS-Ca²⁺-Mg²⁺ buffer containing 1 mg/ml sulfo-NHS-SS-biotin for 1 h at 4 °C. Non-reacted sulfo-NHS-SS-biotin was quenched by washing cells with cold PBS, pH 8.2, containing 100 mM glycine, 0.1 mM CaCl₂ and 1 mM MgCl₂. Cells were harvested in lysis 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, and 1× Complete Protease Inhibitor Mixture (Roche Applied Science)) followed by sonication and centrifugation at 14,000 × g for 10 min at 4 °C to pellet insoluble material. Equivalent amounts of protein from the cell lysates (2 mg) were used for streptavidin–agarose pulldown (Pierce). Biotinylated proteins were eluted at 50 °C for 30 min into Laemmli SDS-PAGE sample buffer supplemented with 50 mM dithiothreitol and separated on 8% SDS-PAGE gels followed by Western blot analysis using a monoclonal antibody directed to the C terminus of CFTR (R&D Systems) or β-integrin (BD Biosciences). For determining the amounts of total CFTR or β-integrin expression, equivalent amounts of protein from the same lysates (200 μg) were separated on 6% SDS-PAGE gels followed by Western blot with anti-CFTR or β-integrin antibody. As a control for determining that equivalent amounts of protein were used for the streptavidin–agarose pulldown, equal amounts of protein from the cell lysates (50 μg) were separated by SDS-PAGE followed by Western blot analysis for β-actin.

**Phosphorylation of Nedd4-2**—CFPAC-1 (ΔF508) cells were labeled with [32P]orthophosphoric acid (0.5 mCi/ml; Perkin-Elmer Life Sciences) in phosphate-free DMEM supplemented with 10% dialyzed fetal bovine serum, 2 mM l-glutamine, and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) for 10 h at 37 °C. Labeled cells were washed once with DMEM medium and then incubated with or without 50 nM DEX for 48 h. For RU38486, LY294002, or LY303511 treatment, labeled cells were pretreated with 1 μM RU38486, 2 μM LY294002, or 2 μM LY303511 for 12 h followed by a 48-h incubation with the inhibitor as well as 50 nM DEX. After drug treatment, cells were washed twice with ice-cold PBS and then lysed in radioimmuneprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 0.1% (v/v) SDS, 1% (w/v) sodium deoxycholate, and 1× Complete Protease Inhibitory
itor Mixture (Roche Applied Science)) followed by sonication and centrifugation at 14,000 x g for 10 min at 4 °C to pellet insoluble material. Cell lysates were then precleared with protein A-Sepharose (Zymed Laboratories Inc.) for 1 h at 4 °C followed by centrifugation and protein determination using the BCA protein assay kit (Pierce). The resulting lysates were divided into two equal halves, one for determining Nedd4-2 phosphorylation and one for assaying 32P-labeled Nedd4-2 bound to CFTR. The lysates, adjusted to 1 mg of total protein, were immunoprecipitated either with an anti-Nedd4-2 polyclonal antibody (Abcam) or with an anti-CFTR C-terminal antibody (R&D Systems) for 6 h at 4 °C followed by the addition of protein A-Sepharose beads for overnight incubation. Immunoprecipitated proteins were collected by centrifugation at 5000 x g for 1 min at 4 °C, washed 3 times with radioimmune precipitation assay buffer, and eluted in Laemmli SDS-PAGE sample buffer for 30 min at 50 °C. Eluted samples were then subjected to SDS-PAGE on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Phosphorylated proteins were visualized by autoradiography. The incorporation of 32P was quantified using the PhosphorImager and the ImageQuant Version 5.2 (Typhoon 9410 PhosphorImager, GE Healthcare). After phosphorimaging, the blots were subjected to Western blot analysis for Nedd4-2 or CFTR.

**Immunoprecipitation and Western Blotting—**CFTR immunoprecipitation from cell lysates treated with drugs or siRNA was carried out as described above. Two mg of total protein was immunoprecipitated with the anti-CFTR C-terminal antibody and immobilized protein A. After extensive washing with radioimmune precipitation assay buffer, the immunoprecipitated proteins were eluted at 50 °C for 30 min in Laemmli SDS-PAGE sample buffer and separated on 10% SDS-PAGE gels. Separated proteins were transferred to a polyvinylidene difluoride membrane for Western blot analysis for either Nedd4-1 or Nedd4-2. For the CFTR ubiquitination assay, drug- or siRNA-treated cells were lysed in radioimmune precipitation assay buffer supplemented with 10 μM MG132, a proteasome inhibitor (Sigma-Aldrich). After immunoprecipitation with anti-CFTR C-terminal antibody, the immunoprecipitated proteins were eluted, separated on 6% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes. CFTR ubiquitination was detected by Western blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (Santa Cruz Biotechnology). As a control for determining that equivalent amounts of protein were used for the immunoprecipitations, equal amounts of protein from the cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes followed by Western blot analysis for Nedd4-2 or β-actin. All immunoblots were subsequently incubated in stripping solution (Chemicon) followed by Western blot analysis for CFTR in the CFTR immunoprecipitates. Immunoreactive proteins were detected using Immobilon-enhanced chemiluminescence (Millipore), and immunoreactive bands were quantitated by densitometry using the Fujifilm Multi Gauge Version 3.0 software.

**Fluorescence Measurement of Chloride Transport—**Chloride transport was measured using the Cl−-sensitive dye MQAE (N’-[ethoxy-carbonylmethyl]-6-methoxyquinolinium bromide; Invitrogen) as described (24). Before chloride transport measurements, CFPAC-1 pLJ6 and CFPAC-1 cells were grown to 70% confluence in 24-well masked tissue culture plates (Wallac, Finland) and treated with appropriate drugs or siRNAs at 37 °C. Drug-treated or Nedd4-2 siRNA-treated cells were loaded with 10 mM MQAE in culture medium for 8 h at 37 °C. After incubation with the fluorescent probe, MQAE-loaded cells were washed three times with a chloride-rich buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 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 then induced by changing from a chloride-rich buffer to a chloride-free buffer of similar composition with (NO3)−1 as the substituting anion without or with a cAMP-agonist containing 10 μM forskolin (Calbiochem), 500 μM IBMX (Sigma-Aldrich), and 500 μM 8-(4-chlorophenylthio)cAMP (Sigma-Aldrich) to stimulate CFTR. In the presence of chloride, MQAE is caged, and the probe fluorescence is quenched. Upon changing over to a chloride-free, nitrate-containing buffer, a chloride gradient is established which results in the rapid exchange of chloride for nitrate in the cells. Over the first few minutes, the rate of increase in free MQAE fluorescence is proportional to the number of chloride channels at the membrane. Increase in MQAE fluorescence was monitored over 20 min after buffer exchange using a FLUOstar Optima microplate reader (BMG Lab Technologies). Data are presented as the increase in MQAE fluorescence (Ft/Fo), where Ft is the fluorescence at time t, and Fo is the initial fluorescence. The initial rates of chloride transport were calculated from the slopes of the fluorescence curves over the first 180 s.

**Cells, siRNA, and Transfection—**CFPAC-1 (ΔF508) cells were cultured as described above. IB3-1 cells (bronchial; ΔF508/W1282X genotype (25)) were maintained at 37 °C in LHC-8 (Invitrogen) medium in a 5% CO2 environment. SGK1 and scrambled siCONTROL non-targeting siRNA were purchased from Ambion/Applied Biosystems (Austin, TX). Nedd4-2 siRNA directed to the sequence AACACAAACAAGTCA was synthesized and tested as previously described by Snyder et al. (10). The custom-made Nedd4-2, scrambled siCONTROL non-targeting, and lamin A/C siRNA were purchased from Dharmacon RNA Technologies (Lafayette, CO). Small interfering RNA solutions were prepared by mixing serum and antibiotic-free DMEM or LHC-8 medium containing 20 nM SGK1, 200 nM Nedd4-2, scrambled control, or lamin A/C (positive control) siRNA with an appropriate volume of Dharmafect 1 transfection reagent (Dharmacon) per 10-cm2 dish or well of a 24-well plate, according to the manufacturer’s instructions. The siRNA mixture was added to CFPAC-1 or IB3-1 cells, grown to 70% confluence, and then incubated at 37 °C for 48 h. After 48 h of treatment, the siRNA-containing medium was changed to fresh medium. After 24 h cells transfected with SGK1 siRNA were treated with or without 50 nM DEX for 48 h. At 72 h after the initial siRNA treatment, the cells were used for cell surface biotinylation, chloride transport, or immunoprecipitation assays as described above. Total protein lysates (50 μg) from these experiments were also subjected to gel electrophoresis and Western blot analysis for
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**A**

| DEX, nM | WT | ΔF508 |
|---|---|---|
| 0 | 250 KDa | 250 KDa |
| 6 | | |
| 12 | | |
| 25 | | |
| 50 | | |

**B**

| Hrs in CHX | WT | ΔF508 |
|---|---|---|
| 0 | 250 KDa | 250 KDa |
| 1 | | |
| 4 | | |
| 8 | | |

**C**

| DEX, nM | WT | ΔF508 |
|---|---|---|
| 1 μM RU38486 | 250 KDa | 250 KDa |
| 0 | | |
| 6 | | |
| 12 | | |
| 25 | | |
| 50 | | |

**D**

| Inhibitor, μM | LY303511 | LY294002 |
|---|---|---|
| DEX: | + | + |
| WT | + | + |
| ΔF508 | + | + |

**FIGURE 2.** Effects of DEX, RU38486, and LY294002 on expression and retention of WT- and ΔF508-CFTR. 

**A.** dose-dependent increase in total CFTR and cell surface expression induced by DEX. Repaired CFPAC-1 pLJ6 (WT) and CFPAC-1 (ΔF508) cells were incubated at 37°C with indicated concentrations of DEX for 48 h. Cell surface proteins were biotinylated and precipitated using streptavidin beads. Biotinylated and total CFTR were detected by Western blot (WB) analysis. Immunoblots of β-actin on the same cell lysates used for streptavidin precipitation are shown below the CFTR panels. Data points are normalized to the values for β-actin and expressed as the average ± S.D. (n = 3). *, p < 0.05 versus vehicle control. The broken line indicates the level of WT-CFTR expression under the untreated condition. B, enhancement of the plasma membrane half-life of CFTR by DEX. Repaired and CFPAC-1 cells were treated at 37°C with or without 50 nM DEX for 48 h and then incubated at 37°C in the presence of 20 μg/ml cycloheximide (CHX) for the indicated times. At the end of each time period, cells were surface-biotinylated, and biotinylated CFTR proteins were precipitated and analyzed by Western blotting. The relative amounts of biotinylated WT- and ΔF508-CFTR at corresponding time points are shown. Results are plotted as the percentage of the initial biotin labeling at 0 h, normalized to the values for β-actin and expressed as the average ± S.D. (n = 3). *, p < 0.05 versus control at the same time point.

**C.** inhibition by the glucocorticoid receptor antagonist RU38486. Repaired and CFPAC-1 cells were incubated at 37°C for 24 h with 1 μM RU38486 before and during the 48 h incubation with various concentrations of DEX. The cells were then surface-biotinylated, and biotinylated WT and ΔF508-CFTR were detected by Western blotting. Immunoblots for β-actin on the same cell lysates used for precipitation with streptavidin beads are shown below the CFTR panel. D, inhibition by the PI 3-kinase inhibitor LY294002. Repaired and CFPAC-1 cells were incubated at 37°C for 24 h with or without LY294002 (active drug) or LY303511 (inactive drug) before and during the 48 h incubation with 50 nM DEX. The cells were then surface-biotinylated, and biotinylated WT and ΔF508-CFTR proteins were detected by Western blotting. Immunoblots for β-actin on the same cell lysates used for streptavidin precipitation are shown below each CFTR panel. Data points are normalized to the values for β-actin and expressed as the average ± S.D. (n = 3). *, p < 0.05 versus vehicle control. Arrowheads indicate the position of surface biotinylated WT- and ΔF508-CFTR. Immunoblotting data are representative of three independent experiments in C and D.

Nedd4-1 (Santa Cruz Biotechnology), Nedd4-2 (Abcam), lamin A/C (BD Biosciences), SGK1 (Abcam), and β-actin (Sigma).

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

**RESULTS**

Dexamethasone Up-regulates WT- and ΔF508-CFTR Trafficking—Fig. 2A, top panels, shows that treatment of CFPAC-1 (ΔF508) and repaired CFPAC-1 pLJ6 (WT) cells with increasing concentrations of DEX results in dose-dependent increases in the expression of both immature B-band and mature C-band CFTR. Consistently, DEX treatment also increases the cell surface expression of both ΔF508- and WT-CFTR (Fig. 2A, bottom panels). Densitometric analysis shows that in CFPAC-1 cells, DEX treatment at a concentration of 50 nM causes a ~5-fold increase in the level of biotinylated ΔF508-CFTR compared with vehicle control (p < 0.05). Importantly, a similar increment of the level of mature C-band ΔF508-
CFTR is also observed. By contrast, DEX treatment of repaired CFPAC-1 pLJ6 cells results in only an ~2-fold increase in both levels of biotinylated and mature C band CFTR over vehicle control (p < 0.05). We observed virtually no change in the expression levels of β1 integrin in both cell types under these conditions (Fig. 2A, middle panels). Thus, the expression and maturation of ΔF508-CFTR is affected by DEX in a similar manner to the effect of DEX on the WT-CFTR counterpart. Importantly, DEX can correct the trafficking defect of ΔF508-CFTR leading to plasma membrane levels comparable with that of WT-CFTR under the untreated condition (Fig. 2A, broken line). Although significant increases in DEX-induced cell surface expression of ΔF508-CFTR were seen at 8 h (4-fold) and 24 h (7-fold), the greatest increase was observed after a 48-h incubation (see supplemental Fig. S1). We, therefore, standardized the DEX treatment protocol by carrying out a 48-h incubation with fresh DEX introduced at each 24-h interval.

Moreover, to exclude the possibility that internal CFTR might be biotinylated because of access of the biotin to the intracellular space, we performed a similar cell surface biotinylation assay for β1 integrin as described for CFTR (see supplemental Fig. S2). β1 integrin belongs to a family of ubiquitously expressed cell surface receptors and was chosen because, like CFTR, it is a membrane protein with a large pool of immature β1 integrin in the endoplasmic reticulum (ER) (26). Untreated CFPAC-1 pLJ6 and CFPAC-1 cells were cell surface-biotinylated, and biotinylated proteins were extracted using streptavidin beads. The immunoblot shows that, as for CFTR in both cell types, there are two forms of β1 integrin present in the lysate, but only mature β1 integrin is present in the streptavidin pull-down assays. This result is consistent with that previously reported by Rennolds et al. (27) confirming that biotin does not penetrate the cell.

**Dexamethasone Increases Membrane Retention of WT- and ΔF508-CFTR**—Fig. 2B shows that DEX treatment significantly increases the membrane retention of both WT- and ΔF508-CFTR compared with the effect of vehicle control (p < 0.05). Densitometric analysis reveals that in DEX-treated CFPAC-1 (ΔF508) cells, the half-life of the biotinylated ΔF508-CFTR is ~8 h. This is ~8-fold longer than that of the same protein in cells treated with a vehicle control. On the other hand, the half-life of the biotinylated WT-CFTR in DEX-treated CFPAC-1 pLJ6 (WT) cells is ~30 h, which is ~2-fold longer than that of the same protein in control cells. Taken together, these data provide compelling evidence that DEX can correct the trafficking defect of ΔF508-CFTR to a level comparable with that of control WT-CFTR and that DEX can also substantially increase the membrane half-life of the mutant protein. Moreover, DEX treatment appears to affect both WT- and ΔF508-CFTR cell surface and steady-state expression to the same extent. We interpret this observation to indicate that DEX may target a general trafficking pathway for CFTR rather than an alternative pathway specific for the mutant CFTR.

**Dexamethasone-activated ΔF508-CFTR Rescue Is Dependent on PI 3-Kinase**—Activation of SGK1 depends on phosphorylation by PI 3-kinase-dependent protein kinase 1 and 2 (PKD1/2), which are in turn activated by PI 3-kinase (15, 16). Therefore, we investigated whether PI 3-kinase-dependent signaling is required for DEX-dependent activation of CFTR trafficking. Fig. 2D shows that inclusion of the active PI 3-kinase inhibitor LY294002 (right panels), but not the inactive form LY303511 (left panels), abolishes the stimulatory effect of DEX on the levels of both surface-biotinylated WT- and ΔF508-CFTR. These data show that the actions of DEX on both WT- and ΔF508-CFTR trafficking are dependent on a signaling pathway that requires an active PI 3-kinase. Therefore, these data further support the hypothesis that the classical SGK1 signaling pathway is involved in the DEX signaling process.

**Dexamethasone Increases Chloride Transport in CFPAC-1 Cells**—As is shown in Fig. 3A, WT-CFTR-repaired CFPAC-1 pLJ6 cells exhibit a rapid increase in chloride-dependent MQAE fluorescence in response to a cAMP agonist mixture (closed diamonds). By contrast, the CFPAC-1 (ΔF508) cells, grown in the absence of DEX, show no change in MQAE fluorescence when incubated with cAMP agonists (closed triangles). However, when CFPAC-1 (ΔF508) cells are treated with 50 nm DEX for 48 h, cAMP-stimulated chloride transport is observed (closed squares; also Fig. 3B for initial rate, p < 0.05). Western blot analysis of these DEX-treated mutant cells shows that the cell surface protein level of rescued ΔF508-CFTR is nearly equal to that of control WT-CFTR (see Fig. 2A). However, as shown in Fig. 3, A and B, the initial rates and extents of chloride transport mediated by rescued ΔF508-CFTR are only about one-third that of the kinetics of chloride transport mediated by WT-CFTR. We interpret these results to be consistent with the concept that the channel open probability of ΔF508-CFTR is less than that of its WT-CFTR counterpart (28, 29).

To further test whether DEX-activated chloride transport is because of activation of SGK1 signaling, we examined the effects of RU38486 and LY294002 on chloride channel activity in DEX-treated CFPAC-1 (ΔF508) cells. As shown in Fig. 2, C and D, Western blot analysis indicates that virtually no plasma membrane-localized ΔF508-CFTR is expressed in cells under these conditions. As consequently expected, cells treated with either RU38486 or LY294002 before and during DEX treatment exhibit no significant change in MQAE fluorescence in response to a cAMP agonist mixture (Fig. 3B). These data, thus, indicate that CFPAC-1 cells are competent to transport chloride only after exposure to DEX alone but not in combination with either the GR inhibitor or the PI 3-kinase inhibitor. The
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FIGURE 3. Chloride transport in CFPAC-1 cells. A, DEX-enhanced chloride transport. CFPAC-1 (ΔF508) cells were incubated at 37 °C for 48 h with or without 50 nM DEX followed by incubation with MQAE. Chloride transport from repaired CFPAC-1 pLJ6 (WT) cells under no treatment was included to serve as a positive control. Chloride transport from control CFPAC-1 (ΔF508) cells which were loaded with MQAE but not stimulated with a cAMP agonist mixture was included to serve as a negative control. Results are normalized to the fluorescence values at time 0 and expressed as the average ± S.D. (n = 3). B, the summary of initial rates of chloride transport from CFPAC-1 (ΔF508) cells under different experimental conditions is shown. CFPAC-1 cells were treated at 37 °C for 24 h with or without 1 μM RU28486 or 2 μM LY294002 before and during the 48-h incubation with or without 50 nM DEX. Cyclic AMP-stimulated chloride transport was measured as described in A. Chloride transport from untreated CFPAC-1 pLJ6 (WT) cells was included as a positive control. Each bar graph represents the average ± S.D. of three experiments. *, p < 0.05 versus (ΔF508) vehicle control, DEX + RU38486, and DEX + LY294002. CON, control.

observed DEX-induced functional increase in ΔF508-CFTR protein in the plasma membrane is consistent with the concept that DEX stimulates CFTR chloride transport activity by increasing the number of ΔF508-CFTR copies in the plasma membrane.

Dexamethasone Inhibits Formation of the Nedd4-2-CFTR Complex—As shown in Fig. 1, the downstream target of SGK1 is the ubiquitin E3 ligase Nedd4-2. After being phosphorylated by SGK1, Nedd4-2 dissociates from ENaC, thus allowing this protein to be retained in the plasma membrane. Reasoning by analogy, it is possible that Nedd4-2 might also regulate CFTR trafficking through direct interaction. As shown in Fig. 4A, Western blot analysis of CFTR immunocomplexes with anti-Nedd4-2 antibody identifies a band at 110 kDa that is the same size as the one observed with either repaired CFPAC-1 pLJ6 (WT) or CFPAC-1 (ΔF508) cell lysates. However, no such band is observed with control IgG (Fig. 4A, right panel). These results, thus, show that under basal conditions, Nedd4-2 interacts with both forms of CFTR in cells. Fig. 4A, left panel, indicates that interaction with Nedd4 is specific to the isoform 2, as neither WT- nor ΔF508-CFTR interacts with the closely related ubiquitin ligase Nedd4-1.

Fig. 4B, left panel, shows that in CFPAC-1 (ΔF508) cells, as the concentration of DEX increases, the amount of Nedd4-2 co-immunoprecipitated with ΔF508-CFTR significantly decreases in a dose-dependent manner (p < 0.05). Densitometry analysis shows that at a concentration of 50 nM, DEX causes an ~5-fold decrease of Nedd4-2 immunoreactivity compared with that of the control. In contrast, the right panel of Fig. 4B shows that the inhibitory effect of DEX on the interaction between Nedd4-2 and ΔF508-CFTR is completely reversed by the GR antagonist RU38486. Similarly, the inhibitory effect of DEX on formation of the Nedd4-2-ΔF508-CFTR complex is also completely abolished by the active PI 3-kinase inhibitor LY294002 (Fig. 4C, right panel) but not by its inactive form LY303511 (Fig. 4C, left panel). Densitometric analysis reveals that in the presence of LY303511, DEX still causes an approximately 4- or 5-fold decrease of Nedd4-2 immunoreactivity compared with that of the control (p < 0.05). Further Western blot analysis of these CFTR immunoprecipitates with anti-CFTR antibody show a substantial amount of mature C band ΔF508-CFTR concomitantly observed with the reduction of Nedd4-2 immunoreactivity (Fig. 4, B and C, middle panels). Importantly, virtually no significant changes are observed in the expression levels of endogenous Nedd4-2 under these conditions. Similar results are observed for WT-CFTR in repaired cells tested under the same conditions (see supplemental Fig. S3). These data suggest that the mechanism by which DEX promotes ΔF508- and WT-CFTR trafficking and retention at the plasma membrane is to down-regulate the physical interaction between CFTR and Nedd4-2.

Phosphorylation of Nedd4-2—As previously mentioned, Nedd4-2 dissociates from its target proteins after being phosphorylated by SGK1. We, therefore, investigated the phosphorylation state of Nedd4-2 under the above experimental conditions. We metabolically labeled CFPAC-1 (ΔF508) cells with [32P]orthophosphate and then treated them with or without DEX in the absence or presence of RU38486 or LY294002. The lysates of these cells were divided into two equal halves, and each half was immunoprecipitated either with an anti-Nedd4-2 or anti-CFTR C terminus antibody. As shown in Fig. 4D, top left panel, DEX treatment substantially increases the phosphorylation of immunoprecipitated Nedd4-2 by ~6-fold compared with that of the basal control (p < 0.05). By contrast, both
RU38486 and LY294002 treatments abolish the stimulatory effect of DEX on Nedd4-2 phosphorylation (Fig. 4D, top left panel). Subsequent Western blot analysis further shows that equivalent amounts of Nedd4-2 are present in all immunoprecipitates, and virtually no significant changes in the expression of endogenous Nedd4-2 in the lysates are observed (Fig. 4D, middle and bottom left panels). Furthermore, both phosphorimaging and Western blot analysis of immunoprecipitated ΔF508-CFTR show a significant reduction in the level of 32P-labeled Nedd4-2 bound to ΔF508-CFTR in the DEX-treated cells (~6-fold; p < 0.05) compared with those in the control cells or cells treated with DEX in the presence of RU38486 or LY294002.
LY2294002 (Fig. 4D, top and middle right panels). Consistently, in subsequent Western blot analysis with anti-CFTR antibody, mature C band ∆F508-CFTR is only observed in the CFTR immunoprecipitate from the DEX-treated cell lysate (Fig. 4D, bottom right panel). These data, thus, further support our hypothesis that DEX promotes the membrane expression of ∆F508-CFTR by reducing the physical interaction between Nedd4-2 and CFTR upon phosphorylation of Nedd4-2.

Silencing of Endogenous SGK1 Abolishes DEX-activated ∆F508-CFTR Rescue—If, as hypothesized, SGK1 plays a key role in down-regulating Nedd4-2 activity, then we predict that DEX-induced ∆F508-CFTR trafficking by SGK1 siRNA. CFPAC-1 (∆F508) cells were transfected at 37 °C with 20 nM scrambled control or SGK1 siRNA. Cells were then incubated with or without 50 nM DEX for 48 h at 37 °C followed by cell surface biotinylation. After cell lysis biotinylated ∆F508-CFTR was extracted using streptavidin beads and analyzed on an 8% SDS-PAGE gel followed by Western blotting (WB). Total ∆F508-CFTR on the same lysates was also analyzed on a 6% SDS-PAGE gel followed by Western blotting (10% of lysate run on gel). Immunoblots for SGK1 and β-actin on the same cell lysates used for streptavidin extraction are shown below the CFTR panels (2.5% of lysate run on gel). The relative amounts of biotinylated ∆F508-CFTR at the corresponding treatment obtained by densitometry are shown. Data points are normalized to the values for β-actin and expressed as the average ± S.D. (n = 3). *, p < 0.05, scrambled siRNA + DEX versus scrambled siRNA and SGK1 siRNA + DEX. B, effect of SGK1 siRNA on ∆F508-CFTR/Nedd4-2 interaction. CFPAC-1 (∆F508) cells were transfected with scrambled control or SGK1 siRNA and then treated with or without 50 nM DEX as described above. The cell lysates (2 mg) were immunoprecipitated (IP) with an anti-CFTR C-terminal antibody. Nedd4-2 ∆F508-CFTR in the CFTR immunoprecipitates was detected by Western blotting. The immunoblot for Nedd4-2 in the same cell lysates used for immunoprecipitation is shown below (bottom panel). The bar graph shows the relative amounts of Nedd4-2 binding to CFTR immunoprecipitates. Data are normalized for the amount of ∆F508-CFTR immunoprecipitated and expressed as the average ± S.D. (n = 3). *, p < 0.05, scrambled siRNA + DEX versus SGK1 siRNA + DEX. Arrowheads indicate the position of Nedd4-2. Immunoprecipitated mutant ∆F508-CFTR is shown as either a single band (immature B band) or double bands (mature C band and immature B band) depending on the experimental conditions.

FIGURE 5. Effect of SGK1 siRNA on expression of ∆F508-CFTR in CFPAC-1 cells. A, inhibition of DEX-activated ∆F508-CFTR trafficking by SGK1 siRNA. CFPAC-1 (∆F508) cells were transfected at 37 °C with 20 nM scrambled control or SGK1 siRNA. Cells were then incubated with or without 50 nM DEX for 48 h at 37 °C followed by cell surface biotinylation. After cell lysis biotinylated ∆F508-CFTR was extracted using streptavidin beads and analyzed on an 8% SDS-PAGE gel followed by Western blotting (WB). Total ∆F508-CFTR on the same lysates was also analyzed on a 6% SDS-PAGE gel followed by Western blotting (10% of lysate run on gel). Immunoblots for SGK1 and β-actin on the same cell lysates used for streptavidin extraction are shown below the CFTR panels (2.5% of lysate run on gel). The relative amounts of biotinylated ∆F508-CFTR at the corresponding treatment obtained by densitometry are shown. Data points are normalized to the values for β-actin and expressed as the average ± S.D. (n = 3). *, p < 0.05, scrambled siRNA + DEX versus scrambled siRNA and SGK1 siRNA + DEX. B, effect of SGK1 siRNA on ∆F508-CFTR/Nedd4-2 interaction. CFPAC-1 (∆F508) cells were transfected with scrambled control or SGK1 siRNA and then treated with or without 50 nM DEX as described above. The cell lysates (2 mg) were immunoprecipitated (IP) with an anti-CFTR C-terminal antibody. Nedd4-2 ∆F508-CFTR in the CFTR immunoprecipitates was detected by Western blotting. The immunoblot for Nedd4-2 in the same cell lysates used for immunoprecipitation is shown below (bottom panel). The bar graph shows the relative amounts of Nedd4-2 binding to CFTR immunoprecipitates. Data are normalized for the amount of ∆F508-CFTR immunoprecipitated and expressed as the average ± S.D. (n = 3). *, p < 0.05, scrambled siRNA + DEX versus SGK1 siRNA + DEX. Arrowheads indicate the position of Nedd4-2. Immunoprecipitated mutant ∆F508-CFTR is shown as either a single band (immature B band) or double bands (mature C band and immature B band) depending on the experimental conditions.

Consistent with the above Nedd4-2/CFTR co-immunoprecipitation data (Fig. 4B, left panel), Fig. 5B shows that in cells transfected with a scrambled control siRNA, DEX treatment significantly reduces the level of membrane-localized ∆F508-CFTR compared with that observed in cells transfected with a scrambled control siRNA (p < 0.05; Fig. 5A, second panel, third and fourth lanes from the left). These data, thus, support the concept that DEX action on ∆F508-CFTR rescue requires the presence of SGK1, which in turn plays a key role in down-regulating Nedd4-2 activity.

Consistent with the above Nedd4-2/CFTR co-immunoprecipitation data (Fig. 4B, left panel), Fig. 5B shows that in cells transfected with a scrambled control siRNA, DEX treatment significantly reduces the level of membrane-localized ∆F508-CFTR compared with that observed in cells transfected with a scrambled control siRNA (p < 0.05). However, this DEX-induced reduction on the formation of the Nedd4-2/∆F508-CFTR complex is not observed in cells transfected with SGK1 siRNA (p < 0.05). A similar result is also observed in cells transfected with SGK1 siRNA in the absence of DEX treatment. No significant changes are observed in the expression levels of endogenous Nedd4-2 under these conditions. Moreover, subsequent Western blot analysis shows that DEX-induced expression of mature C band ∆F508-CFTR is
only observed in the CFTR immunoprecipitate from the scrambled siRNA-treated cell lysate but not in that from the SGK1 siRNA-treated cell lysate (Fig. 5B, second panel, third and fourth lanes). Taken together with the data shown in Fig. 4, these data clearly indicate that DEX rescues ΔF508-CFTR trafficking through the SGK1/Nedd4-2 signaling pathway (see Fig. 1).

Silencing of Endogenous Nedd4-2 Corrects ΔF508-CFTR Trafficking—If Nedd4-2 in the SGK1/Nedd4-2 signaling pathway is responsible for down-regulating CFTR trafficking, then we hypothesize that loss of endogenous Nedd4-2 should rescue ΔF508-CFTR. As shown in Fig. 6A, left panel (also see supplemental Fig. S4B), siRNA against Nedd4-2 specifically knocks down Nedd4-2 protein expression in CFPAC-1 cells by ~70–80% (p < 0.05; see supplemental Fig. S4A) but not the related protein Nedd4-1. This result is consistent with the data previously reported by Snyder et al. (10). The data also show that Nedd4-2 siRNA causes a significant elevation of membrane-localized ΔF508-CFTR compared with a mock transfection or scrambled negative control siRNA (~2.5-fold; p < 0.05). A positive control siRNA against lamin A/C substantially reduces lamin A/C expression but had no effect on the level of plasma membrane CFTR. Thus, when Nedd4-2 expression is reduced, the amount of ΔF508-CFTR in the plasma membrane increases. Similar results are observed in CF lung epithelial IB3-1 cells transfected with the same set of siRNAs (Fig. 6A, right panels), indicating that this is a general phenomenon rather than a cell line-specific effect.

Moreover, Fig. 6B shows that knockdown of Nedd4-2 using siRNA significantly reduces the level of Nedd4-2 binding to the ΔF508-CFTR immunoprecipitate by ~5.5-fold (p < 0.05). These data are consistent with the reduced endogenous level of Nedd4-2 in the treated lysate (Fig. 6B, third panel). Importantly, mature C-band ΔF508-CFTR is observed in the CFTR...
immunoprecipitate from Nedd4-2 siRNA-treated cell lysate (Fig. 6B, second panel). No change is observed in the expression levels of β-actin under both conditions.

Furthermore, as shown in Fig. 6C, scrambled negative control siRNA has a negligible effect on cAMP-stimulated chloride transport in CFPAC-1 cells. However, when cells were treated with Nedd4-2 siRNA, the chloride transport in CFPAC-1 cells significantly increases by a 5-fold higher initial rate compared with the scrambled negative control (Fig. 6C, inset; \( p < 0.01 \)). Remarkably, the chloride transport activity of ΔF508-CFTR induced by Nedd4-2 siRNA appears to be virtually equivalent to that of DEX treatment alone (Fig. 3A). Taken together, these results not only suggest that Nedd4-2 is necessary for CFTR degradation but also that the absence of Nedd4-2 permits accumulation of functional ΔF508-CFTR in the plasma membrane.

Ubiquitination of ΔF508-CFTR—Together, the above results are consistent with the possibility that ubiquitin ligase Nedd4-2 binds to CFTR and is at least partly responsible for CFTR ubiquitination in CFPAC-1 cells. Consistently, Fig. 7A shows that when the CFTR immunoprecipitates from control cell lysates are subjected to Western blot analysis with an anti-ubiquitin antibody, a distributed high molecular weight signal is specifically observed with sizes ranging from 140 to 200 kDa. By contrast, treatment of cells with DEX results in a significant 5-fold reduction in the distributed ubiquitination signal in the CFTR immunoprecipitate (\( p < 0.05 \); Fig. 7A, left panel). As might be anticipated from the preceding data, this DEX-induced reduction of ΔF508-CFTR ubiquitination is reversed by treatment with the GR antagonist RU38486 or the PI 3-kinase inhibitor LY294002. As shown in Fig. 7A, right panel, similar immunoreactive patterns are detected in all CFTR immunoprecipitates from cell lysates treated with RU38486 or LY294002 alone or in combination with DEX, compared with that from the control lysate. As anticipated by the co-immunoprecipitation data from

FIGURE 7. Ubiquitination of ΔF508-CFTR in CFPAC-1 cells. A, effects of DEX, RU38486, and LY294002 on ΔF508-CFTR ubiquitination. Cells were treated with or without DEX and/or RU38486 or LY284002 as described in Fig. 4. Equivalent amounts of total protein (2 mg) were subjected to immunoprecipitation with a monoclonal anti-CFTR C-terminal antibody or normal mouse IgG and analyzed on a 6% SDS-PAGE gel. ΔF508-CFTR ubiquitination or ΔF508-CFTR in the CFTR immunoprecipitates was detected by Western blotting (WB). The normal mouse IgG did not immunoprecipitate CFTR, thus serving as a negative control. The bar graph is a summary of the above data. Data are normalized for the amount of ΔF508-CFTR immunoprecipitated (IP) and expressed as the average ± S.D. (\( n = 3 \)), \( * \), \( p < 0.05 \). B, effects of SGK1 and Nedd4-2 siRNA on ΔF508-CFTR ubiquitination. Cells were transfected with 20 nm SGK1, 200 nm Nedd4-2, 20 nm scrambled siRNA (SGK1 control), or 200 nm scrambled siRNA (Nedd4-2 control) as described in Figs. 5 and 6. After the siRNA treatment, equivalent amounts of total protein (2 mg) were subjected to immunoprecipitation with a monoclonal anti-CFTR C-terminal antibody or a normal mouse IgG. ΔF508-CFTR ubiquitination or ΔF508-CFTR in the CFTR immunoprecipitates was detected by Western blotting. The bar graph is a summary of the above data. Data are normalized for the amount of ΔF508-CFTR immunoprecipitated and expressed as the average ± S.D. (\( n = 3 \)), \( * \), \( p < 0.05 \), scrambled siRNA versus DEX, or scrambled siRNA versus Nedd4-2 siRNA. Immunoblots for β-actin on the same cell lysate used for immunoprecipitation are shown in A and B (2.5% of lysate run on gel).
Figs. 5 and 6, we predicted that knockdown of either endogenous SGK1 or Nedd4-2 expression by siRNA would either elevate or reduce ΔF508-CFTR ubiquitination, respectively. In the case of SGK1 siRNA, Western blot analysis of ΔF508-CFTR immunoprecipitates shows that knockdown of SGK1 completely blocks the DEX-induced reduction of CFTR ubiquitination (Fig. 7B, top left panel; p < 0.05). By contrast, knockdown of Nedd4-2 by siRNA dramatically reduces the ubiquitination of ΔF508-CFTR (Fig. 7B, top right panel; p < 0.05). Importantly, this result is very similar to the results of DEX treatment in non-transfected cells (Fig. 7A, left panel, third lane) or in cells transfected with scrambled control siRNA (Fig. 7B, left panel, second lane). These results confirm the hypothesis that Nedd4-2 binds to CFTR and acts as a CFTR ubiquitin ligase.

**DISCUSSION**

In the present study we have shown for the first time that the SGK1/Nedd4-2 pathway regulates membrane trafficking of CFTR and its mutant form, ΔF508. Our results can be summarized as follows (see Fig. 1). (i) Globally, the glucocorticoid DEX up-regulates functional expression of both WT- and ΔF508-CFTR by increasing total protein expression, cell expression, and the half-life in the plasma membrane. (ii) Blocking either the GR or PI 3-kinase signaling pathway with RU38486 or LY294002, respectively, blocks DEX effects on membrane trafficking of both CFTR forms. (iii) Co-immunoprecipitation data show that the ubiquitin E3 ligase Nedd4-2, but not the related protein Nedd4-1, interacts with both WT and ΔF508 CFTR. This CFTR-Nedd4-2 association is attenuated by DEX in a dose-dependent manner. (iv) The functional rescue of ΔF508-CFTR by DEX is paralleled by the effect of Nedd4-2 knockdown with siRNA. (v) Disruption of the CFTR-Nedd4-2 complex by DEX or knockdown of endogenous Nedd4-2 results in a dramatic reduction in the amount of ubiquitinated ΔF508-CFTR. (vi) Knockdown of endogenous SGK1 completely reverses DEX action on ΔF508-CFTR rescue. (vii) Similar Nedd4-2 knockdown results are observed in experiments with the CF human airway epithelial IB3-1 cells. The latter data indicate that the knockdown effect is general rather than cell line-specific. Taken together, these data indicate that disruption of the CFTR-Nedd4-2 complex results in an increase in cell surface expression of CFTR. We conclude that the SGK1/Nedd4-2 signaling pathway rescues ΔF508-CFTR by regulating the Nedd4-2/CFTR interaction. We further suggest that Nedd4-2 is, thus, a key inhibitory regulator of CFTR trafficking.

These results for CFTR are in total agreement with the classical sequence of actions described for the regulation of ENaC trafficking by the SGK1/Nedd4-2 signaling pathways, as summarized in Fig. 1. However, one important difference between CFTR and ENaC may be how Nedd4-2 interacts with either of these proteins. Nedd4-2 interacts with ENaC by mean of WW domains on Nedd4-2 and PPXY (PY) motifs on ENaC (9, 30). However, conventional PPXY sequences are not present in CFTR, suggesting that Nedd4-2 can either use unconventional mechanisms to directly bind CFTR or that it indirectly interacts with this protein. There is substantial recent evidence for the ability of Nedd4-2 to interact with target proteins that do not possess a PY motif. Examples of such target proteins include the glutamate transporter EAAC1 (31), the voltage-gated K+ channel Kv1.3 (32), the dopamine transporter (33), the voltage-gated K+ channels KCNQ2/3/5 (34), and the insulin-like growth factor I receptor IGF-IR (35). It has also been shown that the WW domains of Nedd4-2 bind to non-PY motifs such as a phosphoserine or phosphothreonine residue followed by a proline residue ((pS/T)p (36)). Several intracellular loops and domains of CFTR do contain such sequences in which a serine or threonine residue is followed by a proline residue. However, whether these sequences in CFTR are phosphorylated by protein kinase(s) is presently unknown. Alternatively, it is increasingly apparent that the Nedd4/Nedd4-2 family of proteins can also interact with target proteins via an independent adaptor protein. For example, Bsd2p connects the Nedd4-2 Saccharomyces cerevisiae homologue Rsp5p to a metal ion transporter Smf1p (37). This interaction is mediated via interaction between WW domains of Rsp5p and the PY motif on Bsd2p. In another example, Grb10α mediates the interaction between Nedd4 and the insulin-like growth factor I receptor IGF-IR via the C2 domain of Nedd4 and the SH2 domain of mGrb10α (38). The above examples, thus, point to a possible common alternative molecular mechanism that requires the presence of an adaptor protein in E3 protein ligase-mediated ubiquitination of target proteins. Further characterization of CFTR/Nedd4-2 interaction is currently under investigation to determine whether this interaction is direct or involves additional adaptor proteins.

Our data also clearly demonstrate that the underlying mechanism for Nedd4-2 regulation of CFTR involves ubiquitination of the CFTR protein. However, our data additionally show that when Nedd4-2 binding to CFTR is inhibited by either DEX treatment or depletion of endogenous Nedd4-2, a small amount of ubiquitinated ΔF508-CFTR was still detected by Western blotting. This phenomenon is likely attributable not only to the presence of residual Nedd4-2 but also to the action of other E3 ligases that are able to ubiquitinate CFTR for degradation. In fact, CHIP is currently the only other known E3 ubiquitin ligase for CFTR. CHIP has been shown to participate in quality control in the CFTR biosynthetic pathway by targeting misfolded WT- and ΔF508-CFTR in the ER for degradation in the proteasome (39). Importantly, inactivation of CHIP only leads to accumulation of ΔF508-CFTR in the ER (40). By contrast, inhibition of binding of Nedd4-2 to CFTR by DEX treatment leads to an increase in both functional expression and the half-life of the mutant protein (Fig. 2). More importantly, depletion of Nedd4-2 also rescues functional ΔF508-CFTR trafficking (Fig. 6). Thus, it is possible that CHIP may only regulate the CFTR trafficking in the ER, whereas the peripheral trafficking of CFTR may be controlled by Nedd4-2.

However, the subcellular location site of Nedd4-2 interaction with CFTR is yet to be determined. The present data focus only on cell lysates and on plasma membrane CFTR. Because Nedd4-2 has been shown to interact with proteins in various subcellular compartments, including the plasma membrane, Golgi apparatus, and endosomes (41, 42),
Nedd4-2 Interacts with CFTR

Nedd4-2 could, therefore, act anywhere in the CFTR trafficking sequence. Coincidentally, CFTR has also been shown to be readily detectable in ER membranes, ER-Golgi transport intermediates, endosomes, and the plasma membrane (43). These observations, therefore, increase the likelihood for the possible association between Nedd4-2 and CFTR at these locations. Furthermore, Nedd4-2 has been implicated in the regulation of sorting processes at the trans-Golgi network, trafficking to endosomes, and endocytosis of the plasma membrane proteins (44).

Therefore, it might be reasonable to expect that upon interaction between Nedd4-2 and CFTR, various consequences could be possible. For example, Nedd4-2 may ubiquitinate and then target newly synthesized CFTR in the Golgi directly to the lysosome. Alternatively, it may target CFTR at the plasma membrane for endocytosis. Or it may promote translocation of internalized CFTR to multiple vesicle bodies in the lumen of endosomes with subsequent degradation in the lysosome. Each of these mechanisms consequently would lead to a reduction of CFTR at the plasma membrane. Provocatively, our SGK1 knockdown results further suggest that one or all of these possible mechanisms might likely be true for ΔF508-CFTR trafficking in epithelial cells (see Figs. 5 and 7B). These data specifically show that only the mature forms of CFTR at the plasma membrane or at the Golgi level are strongly affected by knockdown of SGK1, resulting in more Nedd4-2 binding and, hence, more ubiquitination. Thus, depletion of Nedd4-2 or disrupting the formation of CFTR-Nedd4-2 complexes would reverse these processes and eventually lead to accumulation of CFTR at the cell surface. This scenario would likely be the case for ΔF508-CFTR, as this mutant protein can be detected in ER-Golgi transport intermediates (45–47).

In conclusion, the current study identifies a mechanism by which the DEX-activated SGK1 signaling pathway up-regulates CFTR and its mutant form, ΔF508, by inhibiting Nedd4-2 the association with CFTR. Importantly, Nedd4-2 appears to be the only ubiquitin E3 ligase known so far that is responsible for down-regulation of CFTR in the post-ER compartments, including the plasma membrane. Finally, we interpret these results to indicate that drugs affecting the SGK1/Nedd4-2 signaling pathway may be promising targets for cystic fibrosis therapeutic development.

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