Serum- and Glucocorticoid-induced Protein Kinase 1 (SGK1) Increases the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in Airway Epithelial Cells by Phosphorylating Shank2E Protein*

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Background: Glucocorticoids increase SGK1, which enhances the abundance of CFTR by an unknown mechanism.

Results: SGK1 phosphorylated Shank2E. Knockdown of endogenous Shank2E or overexpression of a dominant-negative Shank2E mutant inhibited the glucocorticoid-mediated increase in CFTR.

Conclusion: The glucocorticoid-induced increase of cell lysate and membrane CFTR is mediated by phosphorylation of Shank2E.

Significance: This study provides novel mechanistic insights into the pathways that regulate CFTR abundance.

The glucocorticoid dexamethasone increases cystic fibrosis transmembrane conductance regulator (CFTR) abundance in human airway epithelial cells by a mechanism that requires serum- and glucocorticoid-induced protein kinase 1 (SGK1) activity. The goal of this study was to determine whether SGK1 increases CFTR abundance by phosphorylating Shank2E, a PDZ domain protein that contains two SGK1 phosphorylation consensus sites. We found that SGK1 phosphorylates Shank2E as well as a peptide containing the first SGK1 consensus motif of Shank2E. The dexamethasone-induced increase in CFTR abundance was diminished by overexpression of a dominant-negative Shank2E in which the SGK1 phosphorylation sites had been mutated. siRNA-mediated reduction of Shank2E also reduced the dexamethasone-induced increase in CFTR abundance.

Taken together, these data demonstrate that the glucocorticoid-induced increase in CFTR abundance requires phosphorylation of Shank2E at an SGK1 consensus site.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated ion channel that mediates bicarbonate and chloride secretion. Together with epithelial sodium channels and aquaporin water channels, CFTR is one of the central regulators of ion and fluid balance across the apical membrane of epithelial cells (1, 2). In the airway, CFTR maintains the airway surface liquid volume essential for mucociliary clearance of inhaled particulate matter and pathogens and is thus an important component of the innate immune system (3).

Loss-of-function mutations in the CFTR gene cause cystic fibrosis, in which dehydration of the airway surface leads to a failure of respiratory pathogen clearance, the development of chronic lung infections and inflammation, and, ultimately, premature death (1, 2). Cigarette smoke and COPD also decrease the amount of CFTR in the apical membrane of airway epithelial cells, which can cause reduced mucociliary clearance and chronic bacterial infections (4–7).

The synthetic glucocorticoid dexamethasone increases the abundance of CFTR in whole cell lysates as well as in the plasma membrane of human airway epithelial cells (8, 9) and pancreatic epithelial cells (10) by increasing levels of the serum- and glucocorticoid-induced protein kinase 1 (SGK1). Recently, we reported that a reduction of SGK1 expression by siRNA (siSGK1) and inhibition of SGK1 activity by GSK 650394 abrogated the ability of dexamethasone to increase plasma membrane WT CFTR in human airway epithelial cells (9). Moreover, in airway epithelial cells, SGK1 regulates CFTR abundance by inhibiting its endocytic removal from the apical membrane (9). Caohuy et al. (10) demonstrated in pancreatic epithelial (CFPAC-1) cells that SGK1 phosphorylates Nedd4-2, thereby reducing Nedd4-2-mediated ubiquitination and degradation of CFTR. By contrast, in airway epithelial cells, Nedd4-2 does not regulate plasma membrane CFTR (11). As a result, in this study, we investigated the possibility that SGK1 acts on a distinct target in airway cells. Because CFTR does not contain an SGK1 phosphorylation consensus sequence, we assessed whether SGK1 phosphorylates non-canonical phosphorylation sites on CFTR. We also explored the possibility that SGK1 may phosphorylate a CFTR-interacting protein that regulates CFTR intracellular trafficking. Here, we report the identification of a specific Shank2 isoform that is a target of SGK1 phosphorylation and test the hypothesis that Shank2 is required for glucocorticoid-mediated enhancement of CFTR abundance.
**EXPERIMENTAL PROCEDURES**

**Cell Culture**—CFBE41o-cells homozygous for the ΔF508 mutation and stably transduced with WT CFTR (12, 13) were grown in minimal essential medium containing charcoal-stripped fetal bovine serum (10%) as described previously (14). CFBE cells were treated with 50 nM dexamethasone or vehicle (ethanol, 1:20,000-dilution in minimal essential medium) for 4 h prior to experiments. HEK293T cells were used to overexpress FLAG-WT-Shank2E for the Shank2E in vitro phosphorylation and Shank2E-SGK1 coimmunoprecipitation experiments.

**Antibodies**—Mouse anti-human CFTR antibody clone 596 (Cystic Fibrosis Foundation Therapeutics Inc., Chapel Hill, NC) was used to probe for CFTR in Western blot analyses and to immunoprecipitate CFTR for phosphorylation assays. An anti-FLAG rabbit polyclonal antibody (Sigma-Aldrich) was used to immunoprecipitate FLAG-tagged WT and mutant Shank2E for phosphorylation assays. Mouse IgG1 antibody (Millipore Australia, Boronia, Australia) and rabbit IgG1 antibody (P120-101, Bethyl Laboratories, Montgomery, TX) were used as negative controls in immunoprecipitations for the CFTR and Shank2E phosphorylation assays, respectively. Western blot analyses for Shank2E were probed with a polyclonal rabbit anti-human Shank2 antibody (H-150, Santa Cruz Biotechnology, Santa Cruz, CA) against a C-terminal region that is common to all Shank2 isoforms. The specificity of the Shank2 antibody as well as its ability to detect the epithelial isoform Shank2E were confirmed by immunoblotting heterologously expressed Shank2E. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Bio-Rad) were used for Western blot analyses. The antibody dilution for Western blot analyses was 1:1000 for all primary antibodies except for Shank2, which was diluted 1:200, and 1:3000 for secondary antibodies. For immunoprecipitation experiments, 20 μg of each antibody was used.

**RT-PCR for Shank2E**—To determine whether Shank2E is expressed in CFBE cells, RNA was isolated using the RNaseasy kit (Qiagen). 2 μg of purified RNA was subjected to RT-PCR using the RETROscript protocol (Ambion/Invitrogen) following the instructions of the manufacturer. PCR was conducted with 280 ng of cDNA template and custom-designed primers specific to human Shank2E (forward, 5'-GACTCCATTTCAGGTGGCCA-3'; reverse, 5'-GGGGTTGGTATGGCTTGACA-3'). The PCR product was sequenced with an ABI 3730 genetic analyzer (ABI/Invitrogen). Negative controls were reverse transcription without template (NC1) and PCR with 2 μg of CFBE total RNA as template (NC2). In a previous Northern blot study, neither Shank1 nor Shank3 mRNA were identified in the lung (15).

**Shank2E Knockdown with siRNA**—To determine whether the dexamethasone-induced increase in CFTR abundance is mediated by Shank2E, endogenous Shank2E protein levels were reduced with siRNA. CFBE cells were seeded at 100,000 cells/filter on collagen-coated 24-mm Transwell permeable supports (Costar, catalog no. 3412) and grown at an air-liquid interface for 3 days. Cells were transfected with 15 nM siRNA against human Shank2 (Qiagen, catalog no. SI04216891) using HiPerfect transfection reagent (Qiagen). 15 nM AllStars siRNA (Qiagen, catalog no. 1027280), hereafter called siNeg, was used as a negative control. Following transfection, cells were polarized on filters for 3 more days. Knockdown of Shank2E was assessed by Western blot analysis using a Shank2-specific antibody (see above). Apical membrane CFTR was quantified, including appropriate controls, using a cell surface protein biotinylation assay, as described in detail in a recent review by our laboratory (16).

**Plasmid Vectors and Overexpression of Shank2E**—Full-length FLAG-tagged rat Shank2E cDNA cloned into a pcDNA3.1 vector was a gift from Prof. Brian Doctor (17). On the basis of this wild-type construct, we generated a phosphorylation site mutant in which the serine residues of the two SGK1 consensus sites of Shank2E were mutated to alanine. This double mutant (pS477A/c.1429 T→G and p.S1755A/c.5263 T→G) was obtained by in vitro mutagenesis using the QuickChange Multi IVM kit (Stratagene) according to the instructions of the manufacturer. The following mismatch primers were used to introduce the desired point mutations: IVM1-F, 5'-GCAGC-GGCTCTCCTCCCGACTAAGGGG-3'; IVM2-F, 5'-GCAGCAGGTCACAGCTCTCCAATATGGC-3'. The presence of the two mutations as well as the integrity of the rest of the cDNA were verified by DNA sequencing of the entire insert. An empty pcDNA3.1 vector (Invitrogen) was used as a negative control. To assess the effect of WT or mutant Shank2E on CFTR membrane abundance, CFBE cells were seeded at 400,000 cells/well in 6-well plates. On the following day, cells were transfected with 0.8 μg/well WT Shank2E, mut-Shank2E, or an empty vector using Effectene transfection reagent (Qiagen). One day after transfection, cells were seeded on 24-mm Transwell filters (Costar, catalog no. 3412) and grown at an air-liquid interface for 3 days before biotinylation experiments. Apical membrane CFTR was quantified using a cell surface protein biotinylation assay as described previously (16).

**CFTR and Shank2E Immunoprecipitation and in Vitro Phosphorylation Assay**—The protocol used to determine whether recombinant SGK1 (Millipore, Billerica, MA) phosphorylates CFTR and Shank2E was modified from Chappe et al. (18) and Hastie et al. (19) and followed the instructions of the manufacturer for SGK1 (Millipore). For the CFTR phosphorylation assay, 4 × 10^6 CFBE cells were seeded on 75-mm Transwell filters (Costar, catalog no. 3419) and grown in culture for 7 days. For the Shank2E phosphorylation assay, 12 × 10^6 HEK293T cells were seeded on T75 flasks and transfected the next day (at 90% confluence) using Effectene transfection reagent (Qiagen) with 2 μg of FLAG-tagged WT Shank2E or 6 μg of FLAG-tagged Shank2E in which the two SGK1 consensus sequences were mutated (mut-Shank2E), as described above. The day after transfection, cells were cultured in serum-free minimal essential medium and incubated for another 24 h. Before lysis, cells were incubated with kinase inhibitors (5 μM chelerythrine chloride and 500 μM H-89 dihydrochloride hydrate, both from Sigma-Aldrich) for 30 min at 37 °C to reduce basal phosphorylation. Subsequently, cells were washed with cold PBS (pH 7.4) (Invitrogen) and lysed in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, and 20 mM Tris (pH 8)) supplemented with EDTA-free complete protease inhibitor (Roche Diagnostics), 5 μM...
chelerythrine chloride and 500 nM H-89 dihydrochloride hydrate. Cell lysates were centrifuged at 16,000 × g for 15 min at 4 °C. To precipitate CFTR or FLAG-tagged WT-mut-Shank2E, supernatants were added to protein G-agarose beads (Thermo Fisher Scientific, Waltham, MA) either containing CFTR, FLAG, or rabbit IgG control antibody (20 μg of antibody/50 μl). After overnight incubation at 4 °C on a rotor, the agarose beads were washed twice with radioimmunoprecipitation assay buffer and three times with phosphorylation buffer (140 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 0.5 mM CaCl₂, and 10 mM Tris–HCl (pH 7.4)). After the last wash, beads were sucked dry with a 27.5 gauge needle and resuspended in 25 μl of phosphorylation buffer. 10 μl of the suspension was used for Western blot analysis of CFTR or FLAG-tagged WT-mut-Shank2E. For the in vitro phosphorylation assay, beads with immunoprecipitated CFTR or FLAG-tagged WT-mut-Shank2E, in 15 μl of phosphorylation buffer, were incubated with 10 μl of 5X reaction buffer (40 mM MOPS and 1 mM EDTA (pH 7.0)), 10 μl of water, and 25 or 100 ng (as indicated) active or inactive SGK1 (catalog nos. 14-331 and 14-332, Millipore) in 5 μl of dilution buffer (20 mM MOPS, 0.1 mM EDTA, 5% glycerol, 0.01% Brij-35, 0.1% 2-mercaptoethanol, and 1 mg/ml BSA), 15 μl of PKA (8U), or 15 μl of phosphorylation buffer only. Samples were warmed to 30 °C, mixed with 10 μCi [γ-32P]ATP (PerkinElmer Life Sciences, Waltham, MA, catalog no. LU002A100UC), 20 μM MgATP, and 10 μg of BSA in 10 μl of phosphorylation buffer and incubated at 30 °C for 10 min. The reaction was stopped by addition of 1 ml of ice-cold radioimmunoprecipitation assay buffer. Beads were washed twice in ice-cold radioimmunoprecipitation assay buffer and three times in phosphorylation buffer, and phosphorylated proteins were eluted from beads with SDS-PAGE sample buffer (Laemmli buffer + 80 mM DTT) by incubation at 85 °C for 10 min. Radiolabeled proteins (CFTR or FLAG-tagged WT/mut-Shank2E) were separated by SDS-PAGE, transferred to Immobilon P PVDF membranes (Millipore), and exposed to a storage phosphor screen. Phosphorylation was detected with a STORM PhosphorImager (GMI Inc., Ramsey, MN). In addition, experiments were conducted to determine whether SGK1 phosphorylates Shank2E peptides that contain either the first or the second SGK1 consensus site and the same peptides with the phosphoacceptor serine changed to an alanine. Sequences of the peptides were as follows (SGK1 consensus sites are shown in boldface): WT1-Shank2E (YAPGPRRSRPSPSLNRGL), WT2-Shank2E (LPNAGR5RSPSPSLIQ), mut1-Shank2E (YAPGPRRSPALNRLG), and mut2-Shank2E (LPNAGR5RSPAPSILQ). Phosphorylation of these peptides (300 μM each) and 300 μM Crosstide positive control was examined as described above.

Shank2E and SGK1 Coimmunoprecipitation—In the experiments described above, we observed significant phosphorylation of Shank2E in vitro, even in the absence of exogenously added SGK1. Thus, to test the hypothesis that SGK1 coimmunoprecipitates with Shank2E and phosphorylates Shank2E even in the absence of added SGK1, we assessed the ability of the immunoprecipitated complex of Shank2E-SGK1 to phosphorylate the Crosstide peptide in vitro. HEK293T cells were transfected with FLAG-WT-Shank2E, FLAG-WT-Shank2E was immunoprecipitated with the FLAG antibody, and the immunoprecipitated protein was incubated with 10 μCi [γ-32P]ATP (PerkinElmer Life Sciences, catalog no. BLU002A100UC) and 30 μM Crosstide peptide (Millipore) in the presence and absence of 100 nM SGK1 inhibitor GSK 650394 (TOCRIS Bioscience/R&D Systems).

Data Analysis and Statistics—Western blot analyses were quantified using ImageJ software (National Institutes of Health). Statistical analysis was performed with GraphPad Prism version 5.0 for Macintosh (GraphPad Software, San Diego, CA). Mean values were compared using paired Student’s t test or repeated measures analysis of variance followed by Tukey’s test, as appropriate. p < 0.05 was considered significant. Data are expressed as mean ± S.E.

RESULTS

SGK1 Does Not Phosphorylate CFTR—Although CFTR does not have a canonical SGK1 phosphorylation sequence, it remained possible that SGK1 may act at a non-canonical site. Thus, we first tested whether SGK1 can directly phosphorylate CFTR. CFTR was immunoprecipitated from CFBE cells, and the immunoprecipitated proteins were incubated in the presence of [γ-32P]ATP with SGK1 or with PKA, which is known to phosphorylate CFTR (20–22). Although PKA showed robust activity, CFTR was not phosphorylated by SGK1 at a level distinct from that seen with catalytically inactive SGK1 (Fig. 1). Thus, we conclude that CFTR is not itself a substrate for SGK1.

Shank2 Proteins Contain SGK1 Consensus Phosphorylation Sites—An alternative possibility is that SGK1 may act by phosphorylating one of the proteins regulating the intracellular trafficking and assembly of CFTR. We and others have shown that plasma membrane abundance of CFTR is regulated by a number of PDZ proteins, including NHERF1/EBP50, NHERF2, and CFTR-associated ligand (1, 23–36). A common feature of these proteins is the presence of a PDZ (PSD-95, Dlg, ZO-1) domain, a protein–protein interaction module that engages the C terminus of CFTR. Therefore, we surveyed known PDZ partners of CFTR to identify possible SGK1 substrates. One of these partners is Shank2 (37–40), which was first described as an actin-binding PDZ protein enriched in the postsynaptic density of the brain (41, 42). Although multiple Shank2 isoforms have been postulated (15), only three are well characterized. Short (hereafter referred to as “Shank2,” also called CortBP1) and medium-length (ProSAP1) isoforms are found in brain tissue, whereas a longer isoform (here referred to as “Shank2E”) is dominant in liver and kidney epithelia (43) (Fig. 2A). Shank2E contains six N-terminal ankyrin repeats and an SH3 domain, which are absent in the short Shank2 isoform (37, 43). Previous studies have shown that Shank2 interacts with CFTR in the rat colon.
and pancreas as well as in heterologous cells overexpressing both proteins (38, 40) and that heterologous expression of Shank2 in NIH 3T3 cells increased plasma membrane CFTR by 39%, although the increase was not statistically significant (38). Both isoforms contain a shared C-terminal SGK1 consensus site, and Shank2E contains an additional site in the N-terminal region that is not present in the short Shank2 isoform (Fig. 2A).

Shank2E Is Expressed in CFBE Cells—Shank2 isoforms are expressed at the apical membrane in epithelial cells in the pancreas, colon, liver, and kidney (38, 43). However, expression has not yet been characterized experimentally in airway epithelial cells. Thus, studies were conducted to determine whether CFBE cells express Shank2 and/or Shank2E. Immunoblotting cell lysates of CFBE cells with a Shank2 antibody raised against a C-terminal region common to all Shank2 isoforms revealed that the long epithelial isoform, Shank2E, is the predominant isoform expressed in this cell type (Fig. 2A). The expression of Shank2E in CFBE cells was further corroborated by PCR with primers specific for the long isoform, Shank2E (Fig. 2C). The resulting PCR product was sequenced and confirmed to be the long isoform, Shank2E (NM_012309.3). Thus, as shown previously for epithelial cells from other tissues (43), human airway epithelial cells express Shank2E.

Knockdown of Shank2E Reduces the Dexamethasone-mediated Increase in CFTR Abundance—If phospho-Shank2E mediates the dexamethasone-induced increase in plasma membrane and total cell CFTR, then knockdown of Shank2E protein abundance should reduce the ability of dexamethasone to increase CFTR abundance. To test this hypothesis, control and dexamethasone-treated cells were transfected with a nonspecific negative control siRNA (siNeg) or a siRNA targeting human Shank2 (siShank2). Shank2 siRNA reduced the levels of Shank2E in both control and dexamethasone-treated cells (Fig. 3A). Relative to siNeg, siShank2 reduced Shank2E protein levels to 63.6 ± 4.2% in control cells and 60.9 ± 4.9% in dexamethasone-treated cells. Western blot analysis and plasma membrane protein biotinylation studies were conducted to determine whether siShank2 reduced the ability of dexamethasone to increase cellular and plasma membrane levels of CFTR protein. In siNeg-treated cells, dexamethasone increased both total cell and plasma membrane levels of CFTR protein. In siNeg-treated cells, dexamethasone increased both total cell (Fig. 3B) and plasma membrane (Fig. 3C) CFTR compared with cells not treated with dexamethasone. siShank2 did not eliminate the dexamethasone-mediated increase in whole cell lysate CFTR compared with the control (Fig. 3B), but it significantly reduced the amount of dexamethasone-induced whole cell lysate CFTR compared with siNeg. Moreover, siShank2 completely blocked the dexamethasone-mediated increase in plasma membrane CFTR (Fig. 3C), an observation consistent with our hypothesis that SGK1 increases plasma membrane CFTR by phosphorylating Shank2E.

Dexamethasone Does Not Increase Shank2E Protein Levels—To test whether dexamethasone up-regulates Shank2E protein levels and, thereby, increases CFTR abundance, whole cell lysates from control or dexamethasone-treated cells were probed with a Shank2-specific antibody. Western blot analysis of Shank2E levels demonstrated that dexamethasone had no effect on Shank2E protein abundance (Fig. 4) and that SGK1 phosphorylates Shank2E.
does not increase CFTR abundance by increasing Shank2E abundance.

**SGK1 Phosphorylates Shank2E**—In vitro phosphorylation experiments were conducted with full-length Shank2E protein to test the hypothesis that SGK1 phosphorylates Shank2E. Because the Shank2-specific antibody used for the Western blot analyses was not suitable for immunoprecipitation of endogenous Shank2E, HEK293T cells were transfected with either FLAG-tagged WT Shank2E or FLAG-mut-Shank2E, in which the two SGK1 consensus sites had been mutated to detect only SGK1-independent phosphorylation. Subsequently, FLAG-tagged WT and mut-Shank2E were immunoprecipitated with an anti-FLAG antibody, and the immunoprecipitated proteins were incubated in the presence of [γ-32P]ATP with either active or inactive SGK1. As expected, FLAG-WT-Shank2E was phosphorylated in the presence of active SGK1 (Fig. 5A, lane 1), whereas the mutant Shank2E was not (Fig. 5A, lanes 3 and 4).

However, to our surprise, WT Shank2E was also phosphorylated after addition of inactive SGK1 (Fig. 5A, lane 2) or in the complete absence of added SGK1 (Fig. 5A, lane 6). A phosphorylated product was not observed when immunoprecipitation was conducted with a nonspecific IgG (Fig. 5A, lane 5). Thus,
although cells were incubated with protein kinase A and C inhibitors before cell lysis and immunoprecipitation, we suspected that contaminating amounts of SGK1 (or another kinase) were immunoprecipitated along with Shank2E. To test this possibility, we investigated the ability of the SGK1 inhibitor

**FIGURE 5.** Endogenous SGK1 coimmunoprecipitates with and phosphorylates Shank2E. A, FLAG-WT-Shank2E and FLAG-mut-Shank2E were immunoprecipitated (IP) with an anti-FLAG antibody and incubated with 25 ng of recombinant SGK1, either active (aSGK) or inactive (iSGK), in the presence of 32P-labeled ATP. IgG was the negative control for immunoprecipitation (lane 5). Additional controls were immunoprecipitated WT Shank2E and 32P-labeled ATP in the absence or presence of 100 nm SGK1 inhibitor GSK 650394 (SGK inh) without addition of exogenous SGK1 (lanes 6 and 7). Endogenous SGK1 phosphorylated WT Shank2E independently of the addition of exogenous active SGK1 (lanes 1, 2, and 6). This phosphorylation signal was reduced to background levels in the presence of the SGK1 inhibitor (lane 7) as well as with mut-Shank2E (lanes 3 and 4). B, the immunoprecipitated FLAG-WT-Shank2E-SGK1 complex phosphorylates a Crosstide peptide with an SGK1 consensus site (lane 1), and this phosphorylation is inhibited by the SGK1 inhibitor GSK 650394 (lane 2). There was no phosphorylation signal in any of the negative controls (immunoprecipitation with IgG instead of FLAG antibody (lanes 3 and 4) or immunoprecipitation with the FLAG antibody from cell lysate isolated from cells that were not transfected with FLAG-WT-Shank2E (lanes 5 and 6) (n = 2). neg. ctrl., negative control.

**FIGURE 6.** SGK1 phosphorylated the first but not the second SGK1 consensus site of Shank2E. This manual dot blot of a 32P phosphorylation assay shows that active SGK1 phosphorylated a control peptide with an RRXXE SGK1 consensus site (ctrl) as well as a peptide containing the first SGK1 site of WT Shank2E (wt1) but not a peptide with the second SGK1 site of WT Shank2E (wt2) or any of the Ser-to-Ala mutants. Inactive SGK1 and active SGK1 + phosphoric (phos.) acid served as negative controls (second and third rows) (n = 3).

**SGK1 and Shank2E**

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**SGK1 and Shank2E**

Endogenous SGK1 Coimmunoprecipitates with Shank2E—Because of the very low absolute abundance of SGK1 in whole cell lysates and its size of 50 kDa, which coincides with the heavy chain of IgG, immunoprecipitation of SGK1 with Shank2E could not be detected by Western blot analysis. To provide additional support for the conclusion that SGK1 coimmunoprecipitates with FLAG-WT-Shank2E, we examined the ability of the immunoprecipitated FLAG-Shank2E complex to phosphorylate a Crosstide peptide with an SGK1 consensus site. The manual dot blot in Fig. 5B demonstrates that the immunoprecipitated material phosphorylates the SGK1 Crosstide peptide (Fig. 5B, lane 1) and that phosphorylation is inhibited by the SGK1 inhibitor GSK 650394 (Fig. 5B, lane 2). These data are consistent with the studies presented in Fig. 5A and the observation that the Shank2E phosphorylation signal was significantly less in all of the negative controls, which included immunoprecipitation with a nonspecific IgG instead of the FLAG antibody (Fig. 5B, lanes 3 and 4) and immunoprecipitation with the FLAG antibody in cells that were not transfected with FLAG-WT-Shank2E (Fig. 5B, lanes 5 and 6). It is important to note that the phosphorylation signal in Fig. 5B, lanes 5 and 6, represents a nonspecific background and that the intensities of the signals in lanes 5 and 6 were similar to the intensities observed in lanes 2–4 in Fig. 5B. Taken together, the experiments presented in Fig. 5 suggest that endogenous SGK1 coimmunoprecipitates with and phosphorylates FLAG-WT-Shank2E.

SGK1 Phosphorylates the First but Not the Second Consensus Site in Shank2E—Studies were conducted using Shank2E peptides to provide additional support for our hypothesis that SGK1 phosphorylates Shank2E. We examined the ability of SGK1 to phosphorylate four different Shank2E peptides, one for each of the two SGK1 consensus sites found in WT Shank2E as well as two corresponding Shank2E peptides in which the serine in each peptide was mutated to alanine to eliminate the candidate SGK1 phosphoacceptor residue. A manual dot blot of a 32P phosphorylation experiment reveals that active SGK1 phosphorylates a positive control SGK1 Crosstide peptide (Fig. 6, lane 1, ctrl) as well as the peptide containing the first SGK1 consensus sequence of WT Shank2E (Fig. 6, lane 2, wt1) but not a peptide containing the second SGK1 consensus site of WT Shank2E (Fig. 6, lane 3, wt2). Moreover, SGK1 did not phosphorylate the two Shank2E peptides in which the serine to alanine mutation had been made to eliminate the SGK1 consensus sites (Fig. 6, lanes 4 and 5). Inactive SGK1 did not phosphorylate either of the two WT Shank2E peptides, and phosphorylation by the active form was blocked by addition of phosphoric acid (Fig. 6, center and bottom rows). Taken together, these experiments demonstrate that SGK1 phosphorylates the N-terminal, Shank2E-specific SGK1 consensus site but not the second site that is shared with the shorter isoform.
Overexpression of Phosphorylation Site-deficient Mut-Shank2E Abrogates the Dexamethasone-induced Increase in Cell Lysate and Membrane CFTR

To provide additional evidence for a role of Shank2E phosphorylation in regulating cell lysate and plasma membrane CFTR, CFBE cells were transfected with vectors expressing either WT Shank2E, mut-Shank2E lacking the SGK1 phosphorylation site, or with vector alone. Cells were then treated with vehicle or dexamethasone, and total cell CFTR and plasma membrane CFTR were measured. In cells transfected with the empty vector or WT Shank2E, dexamethasone increased CFTR levels in whole cell lysates and in the plasma membrane (Fig. 7). Although WT Shank2E moderately enhanced the effect of dexamethasone on CFTR, overexpression of WT Shank2E did not significantly increase the effect of dexamethasone on CFTR compared with dexamethasone-treated cells transfected with the empty vector. A similar observation was made in NIH 3T3 cells in which Shank2 overexpression tended to increase CFTR, but the effect was not significant (38). By contrast, mut-Shank2E attenuated the effect of dexamethasone on whole cell and plasma membrane CFTR (Fig. 7), suggesting that the SGK1 phosphorylation site-deficient mutant had a dominant-negative effect.

DISCUSSION

The major new finding in this work is that, in human airway epithelial cells, SGK1 selectively phosphorylates the long epithelial isoform of Shank2 (Shank2E) and that the phosphorylation of Shank2E by SGK1 is required for the dexamethasone-mediated increase in cell lysate and plasma membrane CFTR. Several lines of evidence support this conclusion. First, siRNA reduction of WT Shank2E reduced the dexamethasone-induced increase in plasma membrane and cell lysate CFTR compared with control siRNA. Second, GSK 650394, an SGK1 inhibitor, reduced the amount of phosphorylated Shank2E. Third, SGK1 phosphorylated a peptide with the first but not the second SGK1 consensus site of WT Shank2E, and mutating the SGK1 site eliminated SGK1-induced phosphorylation. Fourth, a dominant-negative Shank2E, in which the SGK1 phosphorylation sites had been mutated, inhibited the dexamethasone-induced increase in cell lysate and plasma membrane CFTR. Although phosphorylation of CFTR, notably by PKA, has been shown in a variety of epithelial cells to increase plasma membrane CFTR by inhibiting the endocytic retrieval of CFTR from the membrane and by stimulating the exocytic insertion of CFTR into the plasma membrane (20, 21), we demonstrated here that SGK1 does not increase plasma membrane CFTR by directly phosphorylating CFTR.

Previous studies have shown that both Shank2 isoforms play key roles in regulating the plasma membrane levels of other transport proteins in polarized epithelial cells. For example, a reduction in Shank2E abundance by siRNA reduces the plasma membrane sodium-coupled phosphate transporter in opossum kidney cells grown in a low-phosphate medium (44, 45). In addition, overexpression of Shank2 in PS120/NHE3 cells increases plasma membrane abundance of NHE3 (37, 39). Furthermore, knockdown of Shank2 in Caco-2 intestinal cells by siRNA decreased NHE3 plasma membrane abundance (37). In contrast, a recent study by Jung et al. (46) shows that CFTR membrane density is not altered in intestinal epithelia of Shank2 knockout mice in which all three Shank2 isoforms have been deleted. This suggests that CFTR membrane density is

FIGURE 7. Overexpression of a Shank2E mutant lacking SGK1 consensus sites blocked the Dex-induced increase in cell lysate and plasma membrane CFTR. A, representative Western blot (WB) analyses of cell lysates from control and Dex-treated cells transfected with empty vector, WT Shank2E, or mut-Shank2E and probed with antibodies recognizing CFTR or Shank2. WCL, whole cell lysate. B, summary of cell lysate CFTR protein levels (n = 7). ***, p < 0.001. C, representative Western blot analyses of biotinylated samples from control and Dex-treated cells transfected with empty vector, WT Shank2E, or mut-Shank2E and probed with antibodies against CFTR. D, summary of plasma membrane CFTR protein levels (n = 7). *, p < 0.05. mut-Shank2E had a modest dominant-negative effect, and there was no significant increase in whole cell lysate or membrane CFTR with Dex compared with controls.
regulated by distinct pathways in intestinal and airway epithelia, which might be explained by differences in SGK1 expression or activity in different tissues.

Several studies report that the Shank2-mediated up-regulation of membrane transport proteins depends on the interaction between the C terminus of the transporter and the PDZ domain in Shank2. The C termini of the sodium-coupled phosphate transporter, NHE3, and CFTR interact with Shank2, and this interaction is required for the Shank2-mediated increase in plasma membrane abundance of these transport proteins (17, 37–40). However, this study is notable in that it demonstrates, for the first time, that the Shank2E isoform mediates an increase in cell lysate and plasma membrane CFTR and that this effect is dependent on SGK1 phosphorylation of the first SGK1 consensus site in Shank2E, which is not present in the short Shank2 isoform. Thus, the Shank2-induced increase in plasma membrane CFTR in NIH 3T3 cells heterologously expressing CFTR and Shank2 is unlikely to be mediated by phosphorylation via SGK1 (38).

Additional studies are needed to elucidate how phosphorylation of Shank2E increases cell lysate and plasma membrane CFTR. One possibility is that phospho-Shank2E may stabilize CFTR in the membrane, which would inhibit its endocytotic retrieval and subsequent degradation in the lysosome. This possibility is supported by two lines of evidence. First, in a recent publication, we demonstrated that SGK1 inhibits the endocytotic retrieval of CFTR from the apical membrane of airway epithelial cells (9). Second, Shank2E interacts with dynamin, a GTPase that regulates receptor-mediated endocytosis (47). Thus, the SGK1-induced phosphorylation of Shank2E may interfere with the ability of dynamin to facilitate the endocytotic retrieval of CFTR from the plasma membrane. An alternative possibility is that SGK1-mediated phosphorylation of Shank2E disrupts the interaction between Shank2E and CFTR and, thereby, enhances CFTR-NHERF1 interaction, which would increase plasma membrane CFTR. Indeed, disruption of the CFTR-NHERF1 interaction has been shown to reduce plasma membrane CFTR (28, 29). Finally, although it has been reported that Shank2 reduced PKA-induced phosphorylation of CFTR (38), it is unlikely that such a mechanism is responsible for increased plasma membrane CFTR because, as noted above, PKA-mediated phosphorylation of CFTR increases plasma membrane CFTR (20, 21). Clearly, additional studies, which are beyond the scope of this work, are needed to elucidate how SGK1-mediated phosphorylation of Shank2E increases cell lysate and plasma membrane CFTR in airway epithelial cells.

In conclusion, taken together with a previous manuscript (9), our data demonstrate that dexamethasone increases SGK1, which phosphorylates Shank2E and, thereby, increases cell lysate and plasma membrane CFTR abundance in airway epithelial cells. Thus, we suggest that glucocorticoid therapy and the resulting increase in membrane CFTR in combination with a potentiatior of CFTR activity might have positive effects on lung function in cystic fibrosis patients who have residual CFTR function and others who have reduced CFTR function, including smokers and those with COPD (4–7). Moreover, identification of a drug that up-regulates Shank2E in combination with glucocorticoid therapy would be predicted to increase CFTR activity and benefit patients with reduced CFTR function.

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