Inhibitory Regulation of Cystic Fibrosis Transmembrane Conductance Regulator Anion-transporting Activities by Shank2*

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Accumulating evidence suggests that protein-protein interactions play an important role in transepithelial ion transport. In the present study, we report on the biochemical and functional association between cystic fibrosis transmembrane conductance regulator (CFTR) and a PDZ domain-containing protein Shank2. Exploratory reverse transcription-PCR screening revealed mRNAs for several members of PDZ domain-containing proteins in epithelial cells. Shank2, one of these scaffolding proteins, showed a strong interaction with CFTR by yeast two-hybrid assays. Shank2-CFTR interaction was verified by co-immunoprecipitation experiments in mammalian cells. Notably, this interaction was abolished by mutations in the PDZ domain of Shank2. Protein phosphorylation, HCO3− transport and Cl− current by CFTR were measured in NIH 3T3 cells with heterologous expression of Shank2. Of interest, expression of Shank2 suppressed cAMP-induced phosphorylation and activation of CFTR. Importantly, loss of Shank2 by stable transfection of antisense-hShank2 plasmid strongly increased CFTR currents in colonic T84 cells, in which CFTR and Shank2 were natively expressed. Our results indicate that Shank2 negatively regulates CFTR and that this may play a significant role in maintaining epithelial homeostasis under normal and diseased conditions such as those presented by secretory diarrhea.

Secretory epithelia perform vectorial transport of salt and water molecules by coordinated actions of the transporters expressed in polarized epithelial membranes. One of the key membrane proteins regulating overall fluid movements is the cystic fibrosis transmembrane conductance regulator (CFTR), which itself has an anion-transporting activity (1–3). Aberrant membrane transport caused by either hypo- or hyper-functioning of CFTR, can be detrimental, and may result in life-threatening diseases, such as cystic fibrosis or secretory diarrhea (4, 5). Therefore, the fine regulation of salt and water transport is essential in epithelial and body homeostasis.

Accumulating evidence suggests that protein-protein interaction performs an important role in the regulation of transepithelial ion transport (6). Clustering of ion transporters and associated proteins in microdomains of polarized epithelia can facilitate the effective secretion or absorption of salt and water molecules. In this regard, modular adaptor proteins such as PDZ (PSD-95/discs large/ZO-1) domain-containing proteins have drawn increasing attention due to their ability to form supramolecular complexes (7). We have previously shown that the regulatory interaction between CFTR and Na+H+ exchanger 3 (NHE3) through PDZ-based scaffolds is essential for the coordinated regulation of pancreatic bicarbonate secretion (8). In addition, it was found that a number of membrane transporters and receptors participating in pancreatic fluid formation, such as Na+-HCO3− cotransporters (NBC), purinergic receptors, and secretin receptors have a PDZ-binding motif on their C terminus (9, 10). Therefore, multiple protein interactions through PDZ-based scaffolds are believed to perform a critical role in fluid secretion by pancreatic epithelia and possibly by other CFTR-expressing epithelia.

Recently, a large number of PDZ domain-containing proteins were identified in neuronal cells, especially in the postsynaptic density (PSD) of excitatory synapses. Although limited information is available up to now, in general, organization by PDZ-based scaffolds allows the stable localization of interacting proteins and enhances the rate and fidelity of signal transduction (7). Because both neurons and epithelia share many common features, such as ectodermal origin and polarized intracellular structures, it is predicted that some of these scaffolds are expressed in epithelial cells and that they mediate protein-protein interaction. In this study, we aimed to identify the PDZ domain-containing proteins expressed in secretory epithelia and to further characterize their roles in transepithelial ion transport using integrated molecular, biochemical, and physiological approaches.

In an exploratory RT-PCR, it was found that pancreatic epithelia express the mRNAs of several PDZ domain-containing proteins, including SAP97, PSD-95, and Shank2. Of these, Shank2, an isoform of the recently identified family of multidomain adaptors (11), showed an association with CFTR through its PDZ domain in the yeast two-hybrid system and in the mammalian cells. Measurements in CFTR-expressing NIH 3T3 cells revealed that Shank2 overexpression suppressed the CAMP-induced phosphorylation and activation of CFTR. In ad-
dition, antisense-Shank2 treatment augmented the CFTR-dependent Cl⁻ transport in T84 epithelial cells, in which CFTR and Shank2 are endogenously expressed. The above results indicate that Shank2 mediates inhibitory regulation of CFTR and that this may play an important role in epithelial homeostasis.

EXPERIMENTAL PROCEDURES

Materials—The HA-tagged full-length pcDNA3.1-rShank2/CortBP1 construct has been described previously (12). Rabbit polyclonal anti-Shank2 1136 sera were raised against the SAM region of Shank2. To generate H6 fusion proteins for immunization, aa 1012-1252 of rShank2 was amplified by PCR and subcloned into pSSETB (Invitrogen), and fusion proteins were purified using ProBond resin (Invitrogen). The specificity of the Shank2 antibody was confirmed by immunoblotting. Cosensys expression vectors were constructed as described previously (13). M3A7 monoclonal antibody against the NBD2 domain and 24-1 monoclonal antibody against the C terminus of CFTR were purchased from Upstate Biotechnologies and R&D systems, respectively. NIH 3T3 cells stably expressing CFTR (1) were kindly provided by Dr. Michael J. Welsh (University of Iowa, Iowa City, IA) and were a gift from M. DeBoer's modified Dmold medium containing 10 mm glucose and 10% fetal calf serum. For the stable expression of Shank2, NIH 3T3 cells were transfected with pcDNA3.1-rShank2 constructs and selected with G418. T84 cells originated from human colon epithelial cells were purchased from the American Type Culture Collection (ATCC) and maintained in a 1:1 mixture of Ham's F-12 medium and DMEM supplemented with 5% fetal bovine serum.

Yeast Two-hybrid Analysis—The yeast two-hybrid assay was performed as described earlier (14). The L40 yeast strain harboring reporter genes HIS3 and LacZ, under control of the upstream LexA DNA-binding domain, was used in the assay. To semi-quantify the interaction, HIS3 activity was determined by the percentage of yeast colonies growing on histidine-lacking medium. For pBHA, the yeast BCA strain was co-transformed with the LexA DNA-binding domain construct, cDNA sequences containing about 30 aa of the C terminus of membrane proteins were amplified by PCR and subcloned in-frame into pBHA: nBNCn1 (aa 1190–1218: KYSPEPKSVPTINFEDEKSKYMDATLS), mDra1 (aa 728–757: QEREKRKDFITNNGLRNRQECQYPVETK), hCFTR (aa 1452–1480: HNRNSSKCSSKQIAALKETEEBQDTRL), hNHE3 (aa 800–890: PFRSLNKSVDIFSlQAGDEPQNSP.Est), hvpr receptor (aa 467–495: TSTCQTQVSMTLRVSPPGRSIFSQQEYSLV), and SecReceptor receptor (aa 421–449: SFSNATNPHTXSTKASQRSSFRASII). The pGAD10 (a prey vector, Clontech) constructs containing the P2Z domains of SAP97, PSD-95, and Shank2 were previously described (12, 14, 15).

RT-PCR—RT-PCR analysis was performed using rat pancreatic tissues and isolated pancreatic duct cells as reported previously (8). The primer sequences used for this study were as follows: 1) rSAP102, sense (GTG CCC GCC AAG ACC ACA AAA CTA ACC), antisense (CGC CAC CAC CAA CGC CAC CAC), PCR product 432 bp; 2) rSAP97, sense (GTC GTC GTG CCG GCC TTC ACC ACA CA), antisense (CCT GCC GCC CTG ATG GCG GTA CTG), PCR product 376 bp; 3) rGKAP, sense (GAG GCC GTC CAA AGG TGG CTG GTC), antisense (GGG CCT CCG TTC TCT TTG TCA), PCR product 395 bp; 4) rPSD-93, sense (AAG ACT TCT CCG CCC ACTC A), antisense (TGC CCC GCC GCC TTC ATG G), PCR product 530 bp; 5) rPSD-95, sense (CAG CAC CTT CCT CCA CCC TTA TTA TTT), antisense (ATG GGG AGT TAT GAT GGG GCA GGG GTG AC), PCR product 373 bp; 6) rShank1, sense (TCT TAT AGG GGG AGG TTT GGA CAC AGG A), antisense (GAC GGG GGA CAG CAG CAT CAG CAG), PCR product 366 bp; 7) rShank2, sense (CCG CCC AGC CCC TCT CTC TCA CCA), antisense (CCG CCC TCC CGG ATG CTC AGA CTG AC), PCR product 278 bp; 8) rSCAM, sense (ACA AGG AGC AGC AGG ATT TTG ATT ATT T), antisense (CGG TGG GTC GTG TCG TTG CTT CCA TAG), PCR product 402 bp.

Immunohistochemistry—The pancreas and colon tissues from Sprague-Dawley rats were embedded in OCT (Miles, Elkhart, IN), frozen in liquid N2, and cut into 4-μm sections. Immunohistochemistry of frozen sections was performed as previously reported (8). Briefly, the sections were fixed and permeabilized by incubation in cold methanol for 10 min at −20 °C. Nonspecific binding sites were blocked by incubation for 1 h at room temperature with 0.1 ml of phosphate-buffered saline containing 5% goat serum, 1% bovine serum albumin, and 0.1% gelatin (blocking medium). After blocking, the sections were stained by incubating them with anti-Shank2 1136 and/or anti-CFTR 24-1 antibodies and then treated with fluorescein-tagged secondary antibodies. Images were obtained with a Zeiss LSM 510 confocal microscope.

Immunoblotting and Immunoprecipitation—Pre-clear supernatant or NIH 3T3 lysates were mixed with the appropriate antibodies and incubated overnight at 4 °C in lysis buffer. Immune complexes were collected by binding to mixed protein A/G beads and were washed four times with lysis buffer prior to electrophoresis. The lysis buffer contained (in mM) HEPES 50 (pH 7.4), NaCl 150, EDTA 1, Nonidet P-40 1% (v/v), glycerol 10% (v/v), and the complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). The immunoprecipitates or lysates were suspended in SDS sample buffer and separated by SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes, and the membranes were blocked by a 1-h incubation in Tris-buffered saline immobilized on a blocking solution containing 5% nonfat dry milk. The membranes were then incubated with the appropriate primary and secondary antibodies, and protein bands were detected by enhanced chemiluminescence solutions (Amersham Biosciences). The staining intensities of immunoblots were analyzed using imaging software (MCID version 3.0; Brock University, St. Catharine's, Ontario, Canada).

Site-directed Mutagenesis—Oligonucleotide-directed mutagenesis was performed using QuikChange kit (Stratagene) with pcDNA3.1-rShank2 plasmid according to the manufacturer's protocol. His-10 residue of rShank2 was substituted with different amino acids. The mutagenesis primers were as follows: H109A, CAA TGA AAA TGT CTT (sense) and GGG CGC CAC AGG TGG CAG CAC (antisense), H109R, GAA AAT GTC GTA AAG ATG GGC CGC AGG CAG GTG TGG AAC ATG ATC; and H109Q, GAA AAT GTC GTA AAG ATG GGC CAA AGG CAG GTG TGG AAC ATG ATC. Corresponding antisense primers were also made and used for the PCR reactions to generate plasmids with mutated sequences.

Measurement of Cl⁻/HCO₃⁻ Exchange—Whole cell recordings were performed on CFTR-expressing NIH 3T3 cells after they had been stably transfected with pcDNA3.1-rShank2 or mock plasmids. The pipette solution (in mM) 140 N-methyl-D-glucamine chloride (NMDG-Cl), 5 EGTA, 1 MgCl₂, 1 Tris-ATP, and 10 HEPEs (pH 7.2) and the bath solution contained 140 NMDG-Cl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPEs (pH 7.2) for experiments performed at room temperature (22–25 °C). After establishing the whole cell configuration, CFTR was activated by adding 5 μM forskolin. Currents were digitized and analyzed using an AxoScope 8.1 system and a Digidata 1222A AC/DC Converter. Mean currents were normalized as current densities (pA/μF or pF).

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ment were analyzed using the non-paired Student
1
the indicated number of experiments. The results of multiple experi-
ments were presented as the means ± S.E. of the indicated number of experiments. The results of multiple experiments were analyzed using the non-paired Student’s t test or analysis of variance as appropriate.

RESULTS

Expression of PDZ-based Scaffolds in Pancreatic Epithelia—In previous studies, we reported that interactions between multiple membrane proteins, which have PDZ-binding motif on their C terminus perform important roles in pancreatic bicarbonate secretion (8–10). Therefore, as an initial step, we analyzed the expressions of PDZ domain-containing proteins in pancreatic epithelia by RT-PCR using brain tissue as a positive control. As shown in Fig. 1A, rat pancreatic tissue expressed the mRNAs of SAP97, PSD-95, and Shank2. However, pure pancreatic duct cells isolated by mi-
crodissection showed only the mRNA of Shank2. Occasionally, a faint band of guanylate kinase-associated protein (GKAP) was observed in samples from pancreatic tissue. The expression of Shank2 in pancreatic tissue was further confirmed by immunostaining (Fig. 1B). Immunoblotting showed that Shank2 was localized in the luminal (apical) pole of large and small pancreatic ducts. A weak expression was also detected in the luminal area of pancre-
atic acinar cells (Fig. 1B).

Protein-Protein Interaction between PDZ-based Scaffolds and Membrane Proteins—Possible interactions between the C termini of membrane proteins in pancreatic epithelia and the PDZ domains of SAP97, PSD-95, and Shank2 were screened by the yeast two-hybrid assay. Peptide sequences of class I PDZ-binding motif of the membrane proteins are listed under “Experimental Procedures.” To estimate the intensity of the protein interactions, HIS3 activity was determined by the percentage of yeast colonies growing on histidine-lacking medium to estimate the intensity of protein-protein interactions between the PDZ domains of adapter proteins and the C terminus of membrane proteins. The intensity of protein-protein interactions was determined by the percentage of yeast colonies growing on histidine-lacking medium to estimate the intensity of protein-protein interactions between the PDZ domains of adapter proteins and the C terminus of membrane proteins. After the transfection with the prey and bait vectors, equal amounts of yeast cells were plated on histidine-lacking and histidine-supplemented media. The number of yeast colonies growing on histidine-lacking medium was divided by that of growing on histidine-supplemented medium and expressed as percentage.

Table I: Yeast two-hybrid assay

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<th>Membrane protein</th>
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<th>SAP97, PDZ1–2</th>
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<th>Shank2, PDZ</th>
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<td>%</td>
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<td>0</td>
<td>44</td>
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<td>0</td>
<td>1</td>
<td>2</td>
<td>44</td>
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<tr>
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<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
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<td>Secretin receptor</td>
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* Empty pGAD10 was used as negative control.

Statistical Analysis—Results are presented as the means ± S.E. of the indicated number of experiments. The results of multiple experiments were analyzed using the non-paired Student’s t test or analysis of variance as appropriate.
Inhibition of CFTR Cl⁻ channel activity by Shank2 in NIH 3T3 cells. Cl⁻ channel activities were measured in CFTR-expressing NIH 3T3 cells stably transfected with pcDNA3.1-rShank2/CortBP1 or mock plasmids in the whole cell configuration. A, forskolin treatment produced an inward current in the NMDG-Cl solutions at a holding potential of ~30 mV. B, linear I-V relationships were observed when a ramp pulse from ~50 mV to +50 mV was applied at peak current. C, peak currents were normalized as current densities (pA/pF) and the results of 8 (Mock) and 10 (Shank2) experiments are summarized in panel C.

Recently, it was found that CFTR plays an important role in transepithelial HCO₃⁻ transport by regulating Cl⁻/HCO₃⁻ exchange (2, 19). Moreover, reduced HCO₃⁻ secretion by defective CFTR-dependent HCO₃⁻ transport has been suggested to be an important pathological mechanism in mutant CFTR-induced respiratory and pancreatic diseases (21, 22). Therefore, Cl⁻/HCO₃⁻ exchange activities were measured in NIH 3T3 cells by estimating the pH increase due to Cl⁻ removal from HCO₃⁻-buffered perfusate. As reported earlier (2), the Cl⁻/HCO₃⁻ exchange activities of CFTR-expressing NIH 3T3 cells were highly increased by forskolin stimulation (Fig. 4B), but those of CFTR-non expressing cells were unchanged (Fig. 4A). The basal and forskolin-stimulated activities of CFTR-expressing NIH 3T3 cells were 0.121 ± 0.026 ΔpH unit/min and 0.865 ± 0.150, respectively. In Shank2-overexpressing cells, basal activity (0.079 ± 0.018, p = 0.22) was not significantly changed compared with mock transfected cells. However, similar to the results of whole cell Cl⁻ currents, Shank2 overexpression decreased the cAMP-activated Cl⁻/HCO₃⁻ exchange (0.398 ± 0.093, p = 0.02) in CFTR-expressing NIH 3T3 cells (Fig. 4D).

Molecular Mechanisms Responsible for the Decreased CFTR Activity—Two possibilities were examined to explain the underlying molecular mechanisms of inhibitory effects of Shank2 on CFTR-dependent anion transporting activities. The first possibility involves the reduced membrane expression of CFTR protein. Because Shank2 is known to be associated with cytoskeletal proteins (11), it may affect sorting, trafficking, or the endocytotic recycling of CFTR protein. Membrane proteins were biotinylated and harvested at the designated times and

FIG. 2. Immunoprecipitation of Shank2 and CFTR in NIH 3T3 cells. A, proteins from CFTR-expressing NIH 3T3 cells stably transfected with pcDNA3.1-rShank2/CortBP1 or mock plasmids were precipitated with anti-Shank2 antibody, and then blotted with the anti-CFTR M3A7 antibody. B, His-109 of rShank2 (position øB1 in the class I PDZ domain) was substituted with Arg, Gln, and Ala, and Shank2-CFTR binding was measured by immunoprecipitation (IP). Not that IP in panel B was performed using a different antibody order from that in panel A. In all immunoblotting experiments, 100 µg of protein was loaded into each lane, and IP was performed using a total of 1 mg of lysate.

FIG. 3. Inhibition of CFTR Cl⁻ channel activity by Shank2 in NIH 3T3 cells. Cl⁻ channel activities were measured in CFTR-expressing NIH 3T3 cells stably transfected with pcDNA3.1-rShank2/CortBP1 or mock plasmids in the whole cell configuration. A, forskolin treatment produced an inward current in the NMDG-Cl solutions at a holding potential of ~30 mV. B, linear I-V relationships were observed when a ramp pulse from ~50 mV to +50 mV was applied at peak current. C, peak currents were normalized as current densities (pA/pF) and the results of 8 (Mock) and 10 (Shank2) experiments are summarized in panel C.

It has been reported that the first histidine residue of the second α-helix of PDZ domain (position øB1, His-109 in rShank2) plays an important role in class I PDZ interaction by forming a strong hydrogen bond between its N-3 nitrogen and the hydroxyl group of the −2 serine/threonine residue of the ligand (18). To verify the importance of the PDZ-domain in CFTR-Shank2 interaction, we substituted His-109 with two second α-helix of PDZ domain (position øB1, His-109 in rShank2) with two other amino acids containing nitrogen side chain (H109R and H109Q) and with a hydrophobic amino acid (H109A) and then measured the protein-protein interaction by IP. As shown in Fig. 2B, CFTR-Shank2 interactions were abolished in H109R and H109A mutants by 78 ± 8% and 96 ± 3%, respectively (n = 4). Therefore, it was concluded that PDZ-based interactions are required for CFTR-Shank2 binding.

Inhibition of CFTR-dependent Anion Transport by Shank—It is well known that CFTR protein has a cAMP-activated Cl⁻ channel function (1). Thus, the cAMP-activated chloride channel activities of CFTR-expressing NIH 3T3 cells were measured in the whole cell configuration after they had been stably transfected with pcDNA3.1-rShank2 or mock plasmids. Treatment with the adenyl cyclase activator forskolin produced a large inward current in NMDG-Cl solutions with linear I-V relationships (Figs. 3, A and B). Further treatment with 5-nitro-2-(3′-phenylpropylamino)benzoic acid (30 µM) inhibited this current by 94 ± 3% (not shown). These characteristics were in line with previous observations of CFTR Cl⁻ currents (2). Interestingly, Shank2 overexpression decreased the CFTR current densities (pA/pF) by 53% (Fig. 3B). Therefore, it was concluded that PDZ-based interactions are required for CFTR-Shank2 binding.

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Molecular Mechanisms Responsible for the Decreased CFTR Activity—Two possibilities were examined to explain the underlying molecular mechanisms of inhibitory effects of Shank2 on CFTR-dependent anion transporting activities. The first possibility involves the reduced membrane expression of CFTR protein. Because Shank2 is known to be associated with cytoskeletal proteins (11), it may affect sorting, trafficking, or the endocytotic recycling of CFTR protein. Membrane proteins were biotinylated and harvested at the designated times and
then blotted with anti-CFTR M3A7 antibody (Fig. 5A). In contrast to our expectations, Shank2 overexpression showed a tendency to increase the membrane expression of CFTR by 39 ± 28% (p = 0.11, at 2 h after biotinylation) and to extend the half-life of the biotinylated CFTR protein from 17.2 to 25.4 h (p = 0.13, Fig. 5B), although neither of them reached the statistical
The expression pattern of Shank2 was determined immunohistochemically by double staining with anti-Shank2 and anti-CFTR antibodies. The expression of Shank2 was observed in samples from colonic mucosa than those from the pancreatic tissue, possibly due to the higher amount of CFTR protein in the colonic mucosa than in those from the pancreatic tissue (each performed with 2 mg of protein). It was concluded that the down-regulation of CFTR activity by Shank2 was possibly due to the reduced cAMP-induced phosphorylation as well as the basal phosphorylation. Hence, it was hypothesized that Shank2 overexpression decreased the cAMP-induced phosphorylation of CFTR.

**CFTR-Shank2 Interaction in Colonic Epithelia**—Either hyper-or hypofunction of CFTR perturbs the epithelial homeostasis (4, 5). Concerning the inhibitory regulation of Shank2 on CFTR, colonic epithelia will be the most relevant tissue to examine the physiological role of Shank2, where hyper-functioning of CFTR causes severe life-threatening conditions such as cholera (5, 24). Initially, we investigated the expression of Shank2 in rat colonic mucosa. As shown in Fig. 6A, Shank2 was expressed in colonic mucosa, in fact, higher amounts of CFTR-Shank2 IP products were observed in samples from colonic mucosa than in those from the pancreatic tissue, possibly due to the higher amount of CFTR protein in the colonic mucosa. The expression pattern of Shank2 was determined immunohistochemically by double staining with anti-Shank2 and anti-CFTR antibodies (Fig. 6B). In general, colonic surface epithelia absorb fluids and electrolytes, whereas crypt cells secrete (5). Shank2 was expressed in both surface and crypt cells, although CFTR was expressed principally in crypt cells. As was found in pancreatic epithelia (Fig. 1B), Shank2 was observed in the luminal area of colonic epithelia. Therefore, merged images in cross sections and in longitudinal sections showed that Shank2 and CFTR were highly co-localized in the luminal area of crypt cells (Fig. 6B).

To determine whether the Shank2-mediated inhibition of CFTR has physiological relevance, we attempted to remove Shank2 from colonic T84 cells by stably transfecting antisense-hShank2 clones. As shown in Fig. 7A, antisense-Shank2 treatment caused an average reduction of 79 ± 8% in Shank2 protein expression in T84 cells. We next examined the effects of Shank2 loss on CFTR activity by whole cell patch-clamp studies. A summary of the Cl− channel activities evoked by forskolin (5 μM) and peak currents were normalized as current densities. C, representative traces of I-V relationships (step pulse from −120 mV to +120 mV with 20-mV intervals) obtained from cells having median value of each group. **, p < 0.01; difference from mock.
ward current of $-19.3 \pm 2.9$ pA/pF at a $-40$ mV holding potential in mock transfected cells, and this value was increased to $-37.3 \pm 3.7$ pA/pF ($p < 0.01$) in antisense-hShank2-transfected cells (Fig. 1B). Traces of I-V relationships obtained from cells having median value of each group are shown in Fig. 7C. Forskolin treatment evoked an inward current in NMDG-Cl solutions with linear I-V relationships. In addition, an average inhibition of 91% of this current by glibenclamide (100 $\mu$M) demonstrated that most of the observed currents were induced by CFTR.

**DISCUSSION**

Assembly of specific proteins at the microdomains of intracellular regions is powerful machinery that enables the cell to function properly as well as to form unique subcellular structures. One of the critical components of these protein complexes is the modular adaptor proteins. Recently, a family of PDZ-based adaptors has been identified by several investigators and named independently as Shank, CortBP, or ProSAP (11, 25, 26). Subsequent studies revealed that the Shank family of proteins has many binding partners especially in the PSD of excitatory synapses (12). For example, Shank interacts directly with GKAP and Homer, thus potentially bridging the NMDA receptor and the metabotropic glutamate receptors in neurons (27). However, the results of present study demonstrate that the role of Shank as a molecular scaffold is not limited to the neuronal systems.

The most notable finding in this study is the inhibition of CFTR activity by Shank2. CFTR-dependent Cl$^-$ channel activities and Cl$^-$/HCO$_3^-$ exchange activities were decreased by Shank2 overexpression in CFTR-expressing NIH 3T3 cells (Figs. 3 and 4). In addition, cAMP-induced phosphorylation of CFTR was decreased by Shank2 in the heterologous expression system (Fig. 5, C and D). Importantly, loss of Shank2 by antisense-Shank2 treatment increased CFTR-mediated Cl$^-$ channel activities in colonic T84 cells, in which epithelial properties were retained (Fig. 7). All the above findings suggest that Shank2 has a tonic inhibitory effect on the anion-transporting channel activities in colonic T84 cells, in which epithelial properties were retained (Fig. 7).

As to the experiments in T84 cells, we found that there are considerable degrees of heterogeneity of CFTR activity between batches. In fact, we measured the CFTR Cl$^-$ channel activity in T84 cells from three different sources. Among them, a batch obtained directly from ATCC (CCL-248, lot number 2056314) showed the highest CFTR activity in terms of both the frequency of CFTR-positive cells and the amplitude of CFTR Cl$^-$ currents. Therefore, stable transfection of antisense-Shank2 plasmid was introduced to this batch of T84 cells and functional experiments were performed. However, low expression of CFTR protein in T84 cells hampered the further molecular analyses that had been conducted in NIH 3T3 cells. Although the results in heterologous expression system do not always match with native expression system, we speculate that significant analogy exists between these two systems, because expression of Shank2 inhibited CFTR activity in both NIH 3T3 and T84 cells.

The finding that a protein can bind to CFTR and inhibit its activity is reminiscent of the relationship between syntaxin 1A and CFTR (28). However, in several respects, the inhibitory mechanism of Shank2 on CFTR is different from that of syntaxin 1A. For example, syntaxin 1A binds to the N-terminal part of CFTR and is believed to regulate CFTR activity by directly modulating the gating properties or by affecting membrane trafficking of CFTR (29). On the contrary, Shank2 seems to bind to the C-terminal PDZ-binding motif of CFTR (Table I and Fig. 2B) and regulate its anion transporting activities by inhibiting cAMP-induced phosphorylation (Fig. 5). The mechanisms responsible for the reduced cAMP-induced phosphorylation are currently under investigation. A plausible scenario is that Shank2 associates with a phosphatase that can dephosphorylate CFTR. Alternative possibilities include the competition between Shank2 and CFTR-activating scaffolds at the C terminus of CFTR. For example, PDZ-based scaffolds, such as EBP50/NHERF1, E3KARP/NHERF2, or PDZK1/CAP70 have been suggested to activate CFTR by facilitating protein kinase A-induced phosphorylation or by forming active dimer (30–32).

Although the present study sufficiently showed that Shank2 decreased CFTR phosphorylation (Fig. 5), this does not completely rule out other possible mechanisms for the reduced CFTR activity. For instance, Shank2 has a single PDZ domain, hence it can hamper the reported active dimer formation by multiple PDZ domain-containing scaffolds (32). The competitive balance between Shank2-CFTR binding and EBP50-CFTR binding (or E3KARP-CFTR or CAP70-CFTR) may maintain the homeostatic regulation of CFTR.

By the yeast two-hybrid screening, it was found that Shank2 can associate with NHE3 and NBCn1 in addition to CFTR. Notably, all of these transporters are localized in the luminal membrane of pancreatic duct cells and actively participate in transepithelial HCO$_3^-$ transport (8, 10). In fact, we observed that Shank2 binds to NHE3, and that the overexpression of Shank2 blunted the cAMP-induced inhibition of NHE3 activity in the heterologous expression system of PS120 cells (not shown). These findings imply that Shank2 works as a common regulator for modulating ion and fluid transport in the luminal membrane of epithelia, especially as a counterpart of cAMP-evoked signals. Typically, the Shank family of proteins has multiple protein-protein interaction sites and is characterized by multiple ankyrin repeats, an SH3 domain, a PDZ domain, a long proline-rich domain, and a sterile alpha motif (SAM) domain in N terminus to C terminus order. However, isoforms and splicing variants of Shank showed a considerable degree of variability in their domain compositions (11, 13). Although the name of Shank originated from SH3 domain and ankyrin repeats, the major form of Shank2/CortBP1 found and used in this study does not have SH3 or ankyrin repeat domains, but does have the other three domains, including the PDZ domain. Future studies identifying binding partners for these modular domains may reveal more diverse roles of Shank2 proteins in epithelial function.

In conclusion, we found that Shank2 binds to CFTR and tonically inhibits the cAMP-induced activation of CFTR after an integrated search for new PDZ-based scaffolds in epithelial tissues. Because aberrant CFTR activity, especially uncontrolled hyper-functioning of CFTR evokes life-threatening conditions such as diarrhea in cholera infection, the fine regulation of CFTR activity by Shank2 will be important in maintaining epithelial and body homeostasis.

**REFERENCES**

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Regulation of CFTR by Shank2


