Membrane Transport, Structure, Function, and Biogenesis: Inhibitory Regulation of Cystic Fibrosis Transmembrane Conductance Regulator Anion-transporting Activities by Shank2

Joo Young Kim, WonSun Han, Wan Namkung, Ji Hyun Lee, Kyung Hwan Kim, Hyewon Shin, Eunjoon Kim and Min Goo Lee

doi: 10.1074/jbc.M312871200 originally published online December 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M312871200

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 18 of which can be accessed free at http://www.jbc.org/content/279/11/10389.full.html#ref-list-1
Inhibitory Regulation of Cystic Fibrosis Transmembrane Conductance Regulator Anion-transporting Activities by Shank2*

Joo Young Kim†, WonSun Han†, Wan Namkung‡, Ji Hyun Lee‡, Kyung Hwan Kim‡, Hyewon Shin§, Eunjoon Kim§, and Min Goo Lee¶

From the †Department of Pharmacology and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752 and the §Creative Research Center for Synaptogenesis and Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Guseong-dong, Daejeon 305-701, Korea

Accumulating evidence suggests that protein-protein interactions play an important role in transcellular 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 scaffold 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, HCO$_3^-$ transport and Cl$^-$ current of expression of Shank2 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 transcellular 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$^+$/HCO$_3^-$ 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 transcellular 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 multimodular 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-
ditation, 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 pRSETB (Invitrogen), and fusion proteins were purified using ProBond resin (Invitrogen). The specificity of the Shank2 antibody was confirmed by immunoblots. Cos-7 cells expressing heterologous epitelia were purchased from the American Type Culture Collection and selected with G418. T84 cells originated from human colonic epithelial adenocarcinoma and were maintained in Dulbecco’s modified Eagle’s 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. Rabbit polyclonal antibodies (anti-Shank 1136, anti-CFTR 24-1 monoclonal antibody against the C terminus of CFTR) were generated against the COOH-terminal 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 derived from Balb/c mice. The DGFRα African green monkey kidney cells were purchased from the RIBI (Hamilton, MT). The H1102 cell line was a gift from Dr. John Caplan (University of Illinois, Chicago, IL). The HaCaT keratinocyte cell line was kindly provided by Dr. John McCarthy (University of Michigan, Ann Arbor, MI) and was maintained in DMEM supplemented with 5% fetal calf serum.

Yeast Two-hybrid Assay of CFTR—The yeast two-hybrid assay was performed as described above (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 HIS3 detection, colonies were grown on YPD agar plates and replicated on YPD histidine plates.

Regulation of CFTR by Shank2

Introduction—Our previous study showed that Shank2 mediates inhibitory regulation of CFTR and that this may play an important role in epithelial homeostasis. Shank2 is a member of the Shank family of PDZ-domain-containing synaptic protein. Shank2 contains numerous PDZ domains and is highly conserved among mammals. Shank2 is expressed in multiple tissues, including the brain, retina, heart, and lung (9). Shank2 overexpression has been shown to inhibit CFTR-mediated Cl− transport in T84 epithelial cells (10). However, it is unknown whether Shank2 regulates CFTR in other cell types or in vivo. In this study, we investigated whether Shank2 regulates CFTR in other epithelial cells and whether this regulation is conserved among different cell types.

Regulation of CFTR by Shank2

Methods—Regulation of CFTR by Shank2 was investigated in multiple cell types, including T84 epithelial cells, NIH 3T3 fibroblasts, and HaCaT keratinocytes. The general experimental design is shown in Fig. 1C. Initially, we examined whether Shank2 regulates CFTR 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 pRSETB (Invitrogen), and fusion proteins were purified using ProBond resin (Invitrogen). The specificity of the Shank2 antibody was confirmed by immunoblots. Cos-7 cells expressing heterologous epitelia were purchased from the American Type Culture Collection and selected with G418. T84 cells originated from human colonic epithelial adenocarcinoma and were maintained in Dulbecco’s modified Eagle’s 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. Rabbit polyclonal antibodies (anti-Shank 1136, anti-CFTR 24-1 monoclonal antibody against the C terminus of CFTR) were generated against the COOH-terminal 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 derived from Balb/c mice. The DGFRα African green monkey kidney cells were purchased from the RIBI (Hamilton, MT). The H1102 cell line was a gift from Dr. John Caplan (University of Illinois, Chicago, IL). The HaCaT keratinocyte cell line was kindly provided by Dr. John McCarthy (University of Michigan, Ann Arbor, MI) and was maintained in DMEM supplemented with 5% fetal calf serum.

Yeast Two-hybrid Assay of CFTR—The yeast two-hybrid assay was performed as described above (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 HIS3 detection, colonies were grown on YPD agar plates and replicated on YPD histidine plates.

Regulation of CFTR by Shank2

Introduction—Our previous study showed that Shank2 mediates inhibitory regulation of CFTR and that this may play an important role in epithelial homeostasis. Shank2 is a member of the Shank family of PDZ-domain-containing synaptic protein. Shank2 contains numerous PDZ domains and is highly conserved among mammals. Shank2 is expressed in multiple tissues, including the brain, retina, heart, and lung (9). Shank2 overexpression has been shown to inhibit CFTR-mediated Cl− transport in T84 epithelial cells (10). However, it is unknown whether Shank2 regulates CFTR in other cell types or in vivo. In this study, we investigated whether Shank2 regulates CFTR in other epithelial cells and whether this regulation is conserved among different cell types.

Regulation of CFTR by Shank2

Methods—Regulation of CFTR by Shank2 was investigated in multiple cell types, including T84 epithelial cells, NIH 3T3 fibroblasts, and HaCaT keratinocytes. The general experimental design is shown in Fig. 1C. Initially, we examined whether Shank2 regulates CFTR 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.
and Shank2. However, pure pancreatic duct cells isolated by mi-
s were analyzed using the non-paired Student’s t test or analysis of variance as appropriate.

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.

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 using anti-Shank2 antibody M3-7. Shank2 was localized in the luminal pole of the large (left) and small (right) pancreatic duct cells, and that this was absent from the samples incubated with H6-xShank2-(1012–1252) fusion proteins used for immunization (bottom). M, molecular maker.

![Figure 1](https://example.com/figure1.png)

**Table I**

<table>
<thead>
<tr>
<th>Membrane protein</th>
<th>pGAD10, alone</th>
<th>SAP97, PDZ1–2</th>
<th>PSD-95, PDZ1–3</th>
<th>Shank2, PDZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFTR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>NHE3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>NBCn1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>44</td>
</tr>
<tr>
<td>DRA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VIP receptor</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Secretin receptor</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Empty pGAD10 was used as negative control.*
Regulation of CFTR by Shank2

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 \(\alpha\)-helix of PDZ domain (position \#31, 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 ligand (18). To verify the importance of the PDZ-domain in CFTR-Shank2 interaction, we substituted His-109 with two hydrophobic amino acids (H109A) and (H109Q) and with a hydrophobic amino acid (H109A) and then measured the protein-protein interaction. As shown in Fig. 2A, transfection of pcDNA3.1-rShank2 induced expression of Shank2 protein in NIH 3T3 cells (arrowhead). Notably, large amounts of CFTR proteins were detected in anti-Shank2 IP samples from Shank2-transfected cells (Fig. 2A, right). Co-IP experiments using antibodies in reverse order also demonstrated the association between Shank2 and CFTR (see Fig. 2B).

It has been reported that the first histidine residue of the second \(\alpha\)-helix of PDZ domain (position \#31, 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 hydrophobic amino acids (H109A) and (H109Q) and with a hydrophobic amino acid (H109A) and then measured the protein-protein interaction. As shown in Fig. 2A, transfection of pcDNA3.1-rShank2 induced expression of Shank2 protein in NIH 3T3 cells (arrowhead). Notably, large amounts of CFTR proteins were detected in anti-Shank2 IP samples from Shank2-transfected cells (Fig. 2A, right). Co-IP experiments using antibodies in reverse order also demonstrated the association between Shank2 and CFTR (see Fig. 2B).

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 \(\mu\)M) inhibited this current by 94 \pm 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. 3C).

Recently, it was found that CFTR plays an important role in transepithelial HCO\(_3\)\(^{-}\) transport by regulating Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchange (2, 19). Moreover, reduced HCO\(_3\)\(^{-}\} secretion by defective CFTR-dependent HCO\(_3\)\(^{-}\} transport has been suggested to be an important pathological mechanism in mutant CFTR-induced respiratory and pancreatic diseases (21, 22). Therefore, Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchange activities were measured in NIH 3T3 cells by estimating the pH, increase due to Cl\(^{-}\} removal from HCO\(_3\)\(^{-}\} buffered perfusate. As reported earlier (2), the Cl\(^{-}\}/HCO\(_3\)\(^{-}\} 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 \pm 0.026 \(\Delta\)pH unit/min and 0.865 \pm 0.150, respectively. In Shank2-overexpressing cells, basal activity (0.079 \pm 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\(^{-}\}/HCO\(_3\)\(^{-}\} exchange (0.398 \pm 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

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 \(\mu\)M) inhibited this current by 94 \pm 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. 3C).

Recently, it was found that CFTR plays an important role in transepithelial HCO\(_3\)\(^{-}\) transport by regulating Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchange (2, 19). Moreover, reduced HCO\(_3\)\(^{-}\} secretion by defective CFTR-dependent HCO\(_3\)\(^{-}\} transport has been suggested to be an important pathological mechanism in mutant CFTR-induced respiratory and pancreatic diseases (21, 22). Therefore, Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchange activities were measured in NIH 3T3 cells by estimating the pH, increase due to Cl\(^{-}\} removal from HCO\(_3\)\(^{-}\} buffered perfusate. As reported earlier (2), the Cl\(^{-}\}/HCO\(_3\)\(^{-}\} exchange activities of CFTR-expressing NIH 3T3 ce...

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 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 \(\mu\)M) inhibited this current by 94 \pm 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. 3C).

Recently, it was found that CFTR plays an important role in transepithelial HCO\(_3\)\(^{-}\) transport by regulating Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchange (2, 19). Moreover, reduced HCO\(_3\)\(^{-}\} secretion by defective CFTR-dependent HCO\(_3\)\(^{-}\} transport has been suggested to be an important pathological mechanism in mutant CFTR-induced respiratory and pancreatic diseases (21, 22). Therefore, Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchange activities were measured in NIH 3T3 cells by estimating the pH, increase due to Cl\(^{-}\} removal from HCO\(_3\)\(^{-}\} buffered perfusate. As reported earlier (2), the Cl\(^{-}\}/HCO\(_3\)\(^{-}\} 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 \pm 0.026 \(\Delta\)pH unit/min and 0.865 \pm 0.150, respectively. In Shank2-overexpressing cells, basal activity (0.079 \pm 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\(^{-}\}/HCO\(_3\)\(^{-}\} exchange (0.398 \pm 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. 4. Inhibition of CFTR-dependent Cl⁻/HCO₃⁻ exchange by Shank2 in NIH 3T3 cells. CFTR-dependent Cl⁻/HCO₃⁻ exchanges were analyzed in NIH 3T3 cells by measuring the increase in intracellular pH (pHᵢ) in response to [Cl⁻]ᵢ removal from the HCO₃⁻-containing perfusate (25 mM HCO₃⁻, continuously gassed with 95% O₂ and 5% CO₂). The Cl⁻/HCO₃⁻ exchange activities of CFTR-expressing NIH 3T3 cells were markedly increased by forskolin stimulation (B), but those of CFTR-non expressing cells were unchanged (A). Shank2 overexpression reduced forskolin-stimulated Cl⁻/HCO₃⁻ exchange (B–D).

Fig. 5. Surface biotinylation and phosphorylation of CFTR in NIH 3T3 cells. A and B, membrane proteins were biotinylated for 30 min and harvested at pre-set times. Biotinylated proteins were resolved by SDS-PAGE and immunoblotted with the anti-CFTR M3A7 antibody. Degradation of surface CFTR protein in pcDNA3.1-rShank2 or mock-transfected cells was estimated treating each band intensity at 2 h as 100%. Results of three experiments are summarized in panel B. C and D, ³²P labeling of CFTR protein was performed in NIH 3T3 cells stimulated with forskolin. Individual phosphorylation levels were compared with the basal level (0 μM forskolin) in mock-transfected cells, and the results of four experiments are summarized in panel D. *, p < 0.05; **, p < 0.01; difference from mock.

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
In general, colonic surface epithelia expressed in colonic mucosa, in fact, higher amounts of CFTR–Shank2 from colonic T84 cells by stably transfecting antisense-SHANK2 treated T84 cells. A, the stable transfection of antisense-SHANK2 reduced SHANK2 protein expression by 79 ± 8% in T84 cells (n = 3). B, summarized results of whole cell Cl− channel recordings in T84 cells taken at a holding potential of −40 mV. Cells were stimulated with 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.

Regulation of CFTR by Shank2

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 CFTR. 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− current recordings taken at a holding potential of −40 mV is presented in Fig. 7B. Interestingly, only a proportion of T84 cells showed significant CFTR Cl− channel activities. We regarded the cells showed the forskolin-stimulated current densities over 5 pA/pF as “CFTR-positive,” and analyzed their values for evaluating the effects of Shank2 expression. The proportion of CFTR-positive cells was not different between the mock and the antisense-Shank2-treated cells (7/24 and 5/14, respectively; p = 0.72 by Fisher’s exact test). In all experiments, the viability of cells was verified by observing volume-sensitive Cl− channel activities evoked by 260 mosM bath solutions after the forskolin treatment. Notably, loss of Shank2 by antisense treatment augmented CFTR currents in T84 cells, in which Shank2 and CFTR are endogenously expressed. Forskolin (5 μM) produced an average in-
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 NMGD-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 system (Fig. 5, A and B). This finding implies that Shank2 blunts the cAMP-induced inhibition of NHE3 activity in the heterologous expression system of PS120 cells (not shown). These findings indicate 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**

10. Park, M., Ko, S. B., Choi, J. Y., Muallem, G., Thomas, P. J., Pushkin, A., Lee,