The Cystic Fibrosis Transmembrane Conductance Regulator Interacts with and Regulates the Activity of the HCO$_3^-$ Salvage Transporter Human Na$^+$-HCO$_3^-$ Cotransport Isoform 3*

Meeyoung Park‡§, Shigeru B. H. Ko‡, Joo Young Choi‡, Gaia Muallem‡, Philip J. Thomas‡, Alexander Pushkin¶, Myeong-Sok Lee‖, Joo Young Kim, Min Goo Lee**, Shmuel Muallem‡ ‡‡, and Ira Kurtz¶**

*From the ‡Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, the ¶Department of Medicine, Division of Nephrology, UCLA, Los Angeles, California 90095, the ‖Department of Biological Sciences, Sookmyung Women's University, Seoul 140-742, Korea, and the **Department of Pharmacology and Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-752, Korea*

Cystic fibrosis transmembrane conductance regulator (CFTR) regulates both HCO$_3^-$ secretion and HCO$_3^-$ salvage in secretory epithelia. At least two luminal transporters mediate HCO$_3^-$ salvage, the Na$^+$/H$^+$ exchanger (NHE3) and the Na$^+$-HCO$_3^-$ cotransport (NBC3). In a previous work, we show that CFTR interacts with NHE3 to regulate its activity (Ahn, W., Kim, K. W., Lee, J. A., Kim, J. Y., Choi, J. Y., Moe, O. M., Milgram, S. L., Muallem, S., and Lee, M. G. (2001) *J. Biol. Chem.* 276, 17236–17243). In this work, we report that transient or stable expression of human NBC3 (hNBC3) in HEK cells resulted in a Na$^+$-dependent, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid)- and 5-ethylisopropylamiloride-insensitive HCO$_3^-$ transport. Stimulation of CFTR with forskolin markedly inhibited NBC3 activity. This inhibition was prevented by the inhibition of protein kinase A. NBC3 and CFTR could be reciprocally coimmunoprecipitated from transfected HEK cells and from the native pancreas and submandibular and parotid glands. Precipitation of NBC3 or CFTR from transfected HEK293 cells and from the pancreas and submandibular gland also coimmunoprecipitated EBP50. Glutathione S-transferase-EBP50 pulled down CFTR and hNBC3 from cell lysates when expressed individually and as a complex when expressed together. Notably, the deletion of the C-terminal PDZ binding motifs of CFTR or hNBC3 prevented coimmunoprecipitation of the proteins and inhibition of hNBC3 activity by CFTR. We conclude that CFTR and NBC3 reside in the same HCO$_3^-$-transporting complex with the aid of PDZ domain-containing scaffolds, and this interaction is essential for regulation of NBC3 activity by CFTR. Furthermore, these findings add additional evidence for the suggestion that CFTR regulates the overall trans-cellular HCO$_3^-$ transport by regulating the activity of all luminal HCO$_3^-$ secretion and salvage mechanisms of secretory epithelial cells.

§ Performed all of the experiments in this work as partial fulfillment of Ph.D. dissertation.

‡‡ To whom correspondence should be addressed: University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-9040. Tel.: 214-648-2593; Fax: 214-648-8879; E-mail: SHMUEL.MUALLEM@utsouthwestern.edu.

HCO$_3^-$ concentration is tightly controlled in all biological fluids including fluids secreted by exocrine glands. The ductal systems or their equivalents are the sites of active regulation of HCO$_3^-$ content of the secreted fluids. This is also the site of expression of the cystic fibrosis transmembrane conductance regulator (CFTR)[1] (1–5). The transporters participating in ductal HCO$_3^-$ homeostasis and their regulation are only partially known. Probably, the best results are available in the salivary glands and pancreatic ducts. Active regulation of luminal HCO$_3^-$ concentration and pH$_i$ requires the regulation of both HCO$_3^-$ secretory and absorptive mechanisms. HCO$_3^-$ secretion is believed to occur by HCO$_3^-$ influx across the basolateral membrane mediated by a Na$^+$-HCO$_3^-$ cotransport mechanism (6, 7). The transporter mediating this activity is probably pNBC1, the pancreatic isoform of the electrogenic Na$^+$-HCO$_3^-$ cotransporter family (8, 9). HCO$_3^-$ efflux across the luminal membrane (LM) requires the activity of a Cl$^-$/HCO$_3^-$ exchange mechanism (6, 10, 11) and is dependent on the expression of CFTR both in human and in animal models (11, 12).

In the resting state, secretory glands have to absorb HCO$_3^-$. The transporters involved in HCO$_3^-$ absorption are only beginning to emerge. HCO$_3^-$ influx across the LM is in part the result of Na$^+$/H$^+$ exchange mediated by NHE3 (13, 14). However, in recent studies with the pancreatic (13) and the submandibular gland (SMG) ducts (9), we showed that >50% HCO$_3^-$ absorption (H$^+$ secretion) is mediated by more than one Na$^+$-dependent mechanism that is different from any known NHE isoform. Furthermore, we found that the SMG duct and acinar cells express several splice variants of NBC3 (rat orthologues NBCn1B-D) and used anti-NBC3 antibodies to localize the proteins to the LM (9). Using the perfused duct, we found DIDS-insensitive Na$^+$-dependent HCO$_3^-$ transport activity in the LM of the SMG duct and proposed that one or a combination of the NBC3 splice variants found in this tissue may mediate this activity (9).

The regulation of HCO$_3^-$ transport at rest and the stimulated state is of particular importance because it is aberrant in cystic

fibrosis (12, 15). It is of note that the C terminus of all the HCO_3^- transporters expressed in the LM including CFTR (16), NHE3 (17), hNBC3 (19), and its rat orthologues NBCn1B-D (electroneutral NBC splice variants) (18) end with a PDZ binding motif (DTRL, STHM, and ETSL, respectively). This finding raised the possibility that all of the proteins form a HCO_3^- transport complex held together by scaffold proteins such as EBP50 or PDZK1. CFTR in the complex may serve as a "HCO_3^- sensor" to regulate the activity of the other transporters. Such an arrangement is supported by the tight binding of CFTR (20) and the possible binding of NHE3 (17) to EBP50 and CFTR to PDZK1 (21). In addition, the stimulation of CFTR with protein kinase A inhibited NHE3 activity, and this inhibition required the PDZ binding motif of CFTR (22). These findings prompted us to test the regulatory interaction between CFTR and NBC3. We report here that the expression of hNBC3 in HEK293 cells resulted in a DIDS- and EIPA-insensitive, Na^+-dependent HCO_3^- transport. When expressed in the same cells, the stimulation of CFTR inhibited Na^+-HCO_3^- cotransport by hNBC3. Coimmunoprecipitation (Co-IP) and pull-down assays indicate that the two proteins interact with each other with the aid of PDZ scaffolds. Co-IP and inhibition of hNBC3 activity by CFTR required an intact PDZ binding motif in both proteins. We propose that activated CFTR inhibits NBC3 activity by mutual binding to a scaffolding protein containing PDZ binding domains. In this manner, CFTR is able to regulate another portion of the HCO_3^- secretory/absorptive function of secretory epithelia.

EXPERIMENTAL PROCEDURES

Materials and Solutions—BCECF-AM (2′,7′-bis(2-carboxyethyl-5,6-carboxyfluorescein acetoxymethyl ester), was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-CFTR, clone M3A7, was purchased from Upstate Biotechnology (Lake Placid, NY). A synthetic peptide corresponding to amino acids 1197–1214 was used in rabbits to generate a polyclonal antibody specific for human NBC3 (19). Goat anti-rabbit or anti-mouse antibodies (Jackson Laboratories) were used as the secondary antibodies at a 1:1000 dilution to probe the blots. Cell culture reagents including LipofectAMINE were obtained from Invitrogen. The standard perfusion solution (solution A) contained 145 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES (pH 7.4 with NaOH), and 10 mM glucose. Na^+-free solutions were prepared by replacing Na^+ with *N*-methyl-D-glucamine$^+$. HCO_3^--buffered solutions were prepared by replacing 25 mM NaCl or *N*-methyl-D-glucamine$^+$-Cl with 25 mM $NaHCO_3$ or choline-HCO_3, respectively, and reducing HEPES to 5 mM. HCO_3^--buffered solutions were gassed with 5% CO_2, 95% O_2. The osmolarity of all solutions was adjusted to 310 mosmol with the major salt.

Site-directed Mutagenesis—The QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) was used to generate the ΔC-hNBC3 (ΔESTL). The mutagenesis primers were as follows: antisense 5′-GGT TCA ATT CTA TAA TGA AGT **TTA** AGC ATC CAC GTA TTT C-3′; sense 5′-GAA ATA CGT GGA TGC T**TA A**AC TTC ATT ATA GAA TTG AAC C-3′; and sequencing primer 5′-CGG GAC GTG AAC ACC AAT ATA TAC-3′. Oligonucleotide-directed mutagenesis using the GeneEditor mutagenesis kit (Promega, Madison, WI) was performed in the CFTR expression vector pCMVNot6.2 to delete the C-terminal 4 (ΔDTRL) amino acids. The mutagenesis primer was as follows: 5′-GGA GAC AGA AGA AGA GGT G**TA A**GA TAC AAG GCT TTA GAG AG-3′. Incorporation of all mutations was verified by DNA sequencing.

Transient and Stable Expression in HEK293 Cells—Transient gene transfer was accomplished using LipofectAMINE according to instructions provided by the manufacturer. Stable transfectants of hNBC3 and ΔC-hNBC3 were selected by G418 resistance (800 μg/ml) conferred by a *neoR* gene in the parent vector. After clonal selection by limited dilution, stable transfectants were maintained in normal growth medium supplemented with 400 μg/ml G418.

Animals and Tissue Preparations—Animals were allowed free access to food and water and were studied at 1–2 months of age. Animals were sacrificed by cervical dislocation after ether anesthesia. To harvest the SMG, pancreas, and liver tissues, mice and rats were killed, the abdomen was opened, and the tissues were removed into a dish containing ice-cold high K^+ solution composed of 140 mM KCl, 10 mM HEPES, and 1 mM EDTA with pH 7.0 adjusted with KOH. The tissues were minced

into a fine paste and homogenized by 25 strokes at 1000 rpm with a motor-driven glass-in-Teflon Potter homogenizer. The crude homogenates were centrifuged at $100,000 \times g$ for 15 min, and the pellets were resuspended in high K^+ buffer and immediately used to prepare extract by mixing with a 2× lysis buffer.

Immunoprecipitation and Immunoblotting—These procedures were as described previously (22). Mice, rats, or HEK293 cell lysates (always 400 μg of protein) were mixed with the appropriate antibodies and incubated overnight at 4 °C in lysis buffer. Immune complexes were collected by binding to protein G- or protein A-Sepharose and washing four times with lysis buffer. The immunoprecipitates or lysates (always 40 μg of protein) were suspended in SDS sample buffer and separated by SDS-PAGE electrophoresis. The proteins were detected by incubations with the appropriate primary and secondary antibodies. The anti-hNBC3 antibody was diluted 1:1000 in Tris-buffered saline (20 mM Tris·HCl, pH 7.5, and 137 NaCl), and monoclonal anti-CFTR antibodies were diluted 1:500.

Pull-down Assay—For pull-down experiments, GST-EBP50 was cloned in the pGEX vector and expressed in *Escherichia coli* (Amersham Biosciences GST purification protocol). The vector was used to produce GST alone for the control experiments. Isopropyl-1-thio-β-D-galactopyranoside was used to induce gene expression. The bacteria were disrupted using a combination of freeze-thaw and sonication. The lysate was centrifuged at $18,000 \times g$ for 20 min at 4 °C. The supernatant was dialyzed for 24 h at 4 °C against PBS and incubated with 2 ml of glutathione-Sepharose 4B for 4 h at 4 °C with gentle agitation. The Sepharose beads were washed with 200 ml of PBS, and GST-EBP50 and GST proteins were eluted using glutathione elution buffer and dialyzed against PBS. The proteins were quantitated using SDS-PAGE following by Coomassie Blue staining and Western blotting. 100 μg of GST-EBP50 or GST in 0.5 ml of PBS were mixed with 0.5-ml extracts containing 400 μg of protein prepared from HEK293 cells expressing CFTR, hNBC3, ΔC-hNBC3, CFTR+hNBC3, or ΔC-CFTR+hNBC3. After incubation for 10 h at 4 °C, the solution was mixed with 25 μl of glutathione-Sepharose 4B, incubated for 4 h at 4 °C with gentle agitation, washed 10 times with 1 ml of PBS, eluted with glutathione elution buffer, and analyzed using SDS-PAGE and Western blotting. Primary mouse anti-human CFTR antibody (RDI, Flanders, NJ) was used at a dilution 1:2000, and rabbit anti-hNBC3 antibody (C1) was used at a dilution 1:1000. Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were used at a dilution of 1:20,000.

Intracellular pH Measurement—For the measurement of pH_i in transfected HEK293 cells, glass coverslips with cells attached to them were washed once with solution A and assembled to form the bottom of a perfusion chamber. The cells were loaded with BCECF by a 10-min incubation at room temperature in solution A containing 2.5 μM BCECF-AM, and dye loading was monitored. After dye loading, the cells were perfused with appropriate solutions and pH_i was measured by photon counting using a fluorescence measuring system (Delta Ram, PTI Inc., South Brunswick, NJ). In the case of hNBC3, CFTR or their C-terminal deleted mutants, a GFP-expressing plasmid (Invitrogen), was cotransfected with the constructs, and pH_i measurements were performed with cells expressing GFP. The bath was perfused at a flow rate of 6 ml/min using the desired solutions that were maintained at 37 °C. The fluorescence ratios of 490/440 nm were calibrated by perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin.

Statistical Analysis—Values are expressed as the means ± S.E. The significance of differences between mean values was examined using ANOVA. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Properties of hNBC3 Expressed in HEK293 Cells—At least two HCO_3^- salvage mechanisms have been identified in the LM of the ducts of the secretory glands SMG (9) and pancreas (13) and NHE3 and splice variants of NBC3. Because this membrane expresses CFTR and the scaffold EBP50 (22) and because all of the proteins have a PDZ binding motif in their C terminus, we proposed that CFTR could regulate the overall process of luminal HCO_3^- transport. When stimulated by protein kinase A, CFTR can activate HCO_3^- secretion and in the same time it can inhibit HCO_3^- salvage that take place in the resting state (9, 11, 13). To test this hypothesis, we showed that stimulation of CFTR expressed in heterologous systems (23) and in the native SMG and the pancreatic ducts (11) activates
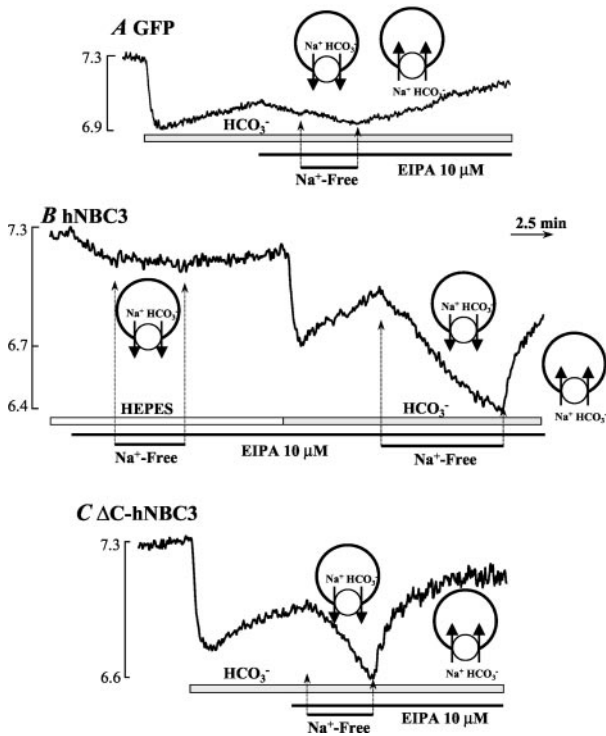
FIG. 1. **Measurement of Na⁺-HCO₃⁻ cotransport in HEK293 cells transfected with hNBC3.** Cells transfected with GFP (*A*), hNBC3 (*B*), or ΔC-hNBC3 (*C*) were incubated in a K⁺-free solution containing 0.1 mM ouabain for 90–120 min before assembly into the perfusion chamber. The cells were perfused with K⁺-free solutions throughout. An *open bar* indicates perfusion with HEPES-buffered solutions, and a *gray bar* indicates perfusion with HCO₃⁻-buffered solutions. The periods of incubation with Na⁺-free solutions are marked by *dotted arrows*. The cells were incubated with 10 μM EIPA as indicated by the *solid lines*. Note that expression of NBC3 resulted in a Na⁺-dependent EIPA-insensitive HCO₃⁻ efflux and influx activity. The models depict the direction of Na⁺-HCO₃⁻ cotransport.

Cl⁻/HCO₃⁻ exchange. Activation of CFTR also inhibited NHE3 activity both in the native pancreatic duct and in heterologous systems (22).

In this work, we continued testing this hypothesis by studying the regulatory interaction between CFTR and NBC3. For this reason, we expressed hNBC3 in HEK293 cells and characterized its activity. hNBC3 and the ΔC-hNBC3 from which the last four-amino acid PDZ binding motif was truncated were expressed either transiently or by isolating stable cell lines expressing the proteins (see "Experimental Procedures"). Similar results were obtained with both methods (data not shown), and thus, the results from both sets of experiments were combined. Fig. 1 shows the basic experimental protocol used to follow NBC activity. Cells were incubated for 90–120 min in a K⁺-free solution containing 0.1 mM ouabain to load them with Na⁺. The cells were maintained in this solution during dye loading and were perfused throughout with K⁺-free solutions to keep the Na⁺ pump inhibited. For controls, HEK293 cells were transfected with GFP only and were equilibrated with a HCO₃⁻-buffered solution. The cells were incubated with 2.5–10 μM EIPA to inhibit all NHE activity and then were exposed consecutively to a Na⁺-free and Na⁺-containing solutions. This resulted in slow rates of cytoplasmic acidification and alkalinization, respectively (Fig. 1*A*), probably because of low endogenous NBC activity (see below). The low NBC activity of this HEK293 clone was suitable for characterizing the activity of the expressed proteins.

Fig. 1*B* shows the activity observed in cells transfected with GFP and hNBC3. Exposing Na⁺-loaded cells incubated
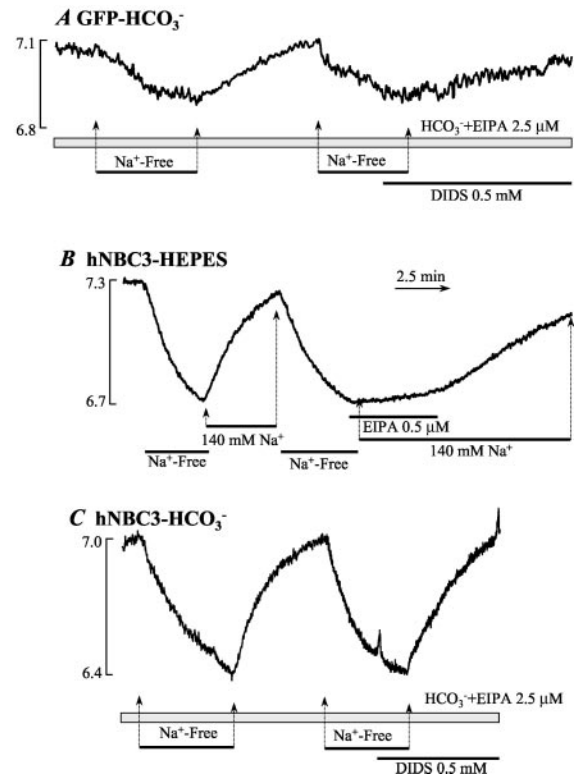

FIG. 2. **Na⁺-HCO₃⁻ cotransport by hNBC3 is resistant to DIDS and EIPA.** Cells transfected with GFP (*A*) or hNBC3 (*B* and *C*) were equilibrated with HCO₃⁻ (*A* and *C*) or HEPES-buffered solutions (*B*). *A* and *C*, the solution also contained 2.5 μM EIPA. The cells were exposed to Na⁺-free solutions to estimate NHE (*B*) or NBC (*A* and *C*) activity before (control period) and after treatment with DIDS. The cells *A* and *C* were treated with 0.5 mM DIDS where indicated by the *solid bar*. The cells in *B* were treated with EIPA as indicated.

in HEPES-buffered solution containing 10 μM EIPA to a Na⁺-free solution had no apparent effect on pH$_i$. This was somewhat unexpected because the expression of hNBC3 in *Xenopus* oocytes showed that hNBC3 could transport both OH⁻ and HCO₃⁻ (19). The difference can be attributed to differential behavior of hNBC3 in the mammalian HEK293 cells and in *Xenopus* oocytes. Alternatively, because hNBC3 transports HCO₃⁻ better than OH⁻, it is possible that the expression levels in HEK293 cells did not reach those in *Xenopus* oocytes and thus did not allow us to see the OH⁻ transport by hNBC3. In this respect, hNBC3 expressed in HEK293 cells behaved like its rat orthologue NBCn1B expressed in *Xenopus* oocytes (18) in that both showed no Na⁺-OH⁻ cotransport.

After equilibration in a HCO₃⁻-buffered medium that also contained 10 μM EIPA, exposing cells expressing hNBC3 to a Na⁺-free medium resulted in rapid cytosolic acidification that was reversed upon re-addition of Na⁺ to the perfusion solution (Fig. 1*B*). Similar to all other cells, HEK293 cells express NHE activity that was almost completely inhibited by 0.5 μM EIPA, indicative of NHE1 activity. This is shown in Fig. 2*B* for cells expressing hNBC3, and similar results were obtained in control cells and cells expressing ΔC-hNBC3. In multiple experiments (at least three at each concentration), EIPA between 0.5 and 100 μM did not inhibit the Na⁺-dependent changes in pH$_i$ illustrated in Fig. 1, *B* and *C*, that were measured in the presence of HCO₃⁻ in cells expressing hNBC3 or ΔC-hNBC3. Hence, again, it seems that hNBC3 behaves differently when expressed in *Xenopus* oocytes and HEK293 cells, because hNBC3 activity was inhibited by 100 μM EIPA in *Xenopus* oocytes (19). The activity of NBCn1B expressed in *Xenopus*
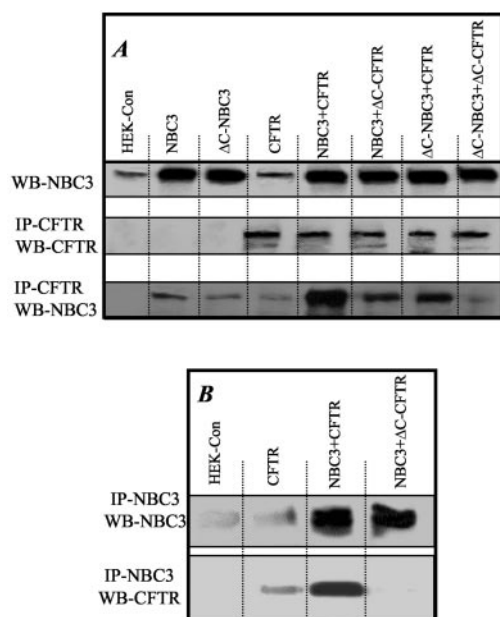
FIG. 3. **Co-IP of CFTR and NBC3 expressed in HEK cells.** *Panel A*, extracts were prepared from control cells (*HEK-Con*) and cells stably expressing hNBC3 or ΔC-hNBC3 and transfected with WT-CFTR or ΔC-CFTR. The cells were used to assay the effect of expression of the CFTR constructs on the expression of NBC3 by Western blot (*upper blot*, 40 μg in each lane) and for immunoprecipitation (*IP*, 400-μg extract at each condition) of CFTR. The immunoprecipitates were analyzed for CFTR (*middle blot*) and hNBC3 (*lower blot*). *Panel B*, 400 μg of extracts from control cells and cells transfected with CFTR only. hNBC3+CFTR or hNBC3+ΔC-CFTR were used to immunoprecipitate hNBC3 and blot for hNBC3 (*upper blot*) or CFTR (*lower blot*).

oocytes was not inhibited by EIPA (18). Finally, Fig. 1*C* shows that ΔChNBC3 behaved similarly to hNBC3. The rates of Na$^+$-dependent HCO$_3^-$ influx in acidified cells were 0.67 ± 0.08 ($n = 17$) and 0.71 ± 0.07 ($n = 6$) pH units/min in cells transfected with hNBC3 and ΔChNBC3, respectively. Therefore, the deletion of the PDZ binding motif of hNBC3 does not affect expression, processing, or activity of the protein.

A feature distinguishing Na$^+$-HCO$_3^-$ cotransport by hNBC3 (19) and its rat orthologues (18) from that by the electrogenic family of NBCs is the insensitivity of the transport to DIDS, because the electroneutral NBCs do not have a canonical DIDS binding motif (18, 19). Therefore, we measured the effect of DIDS on the activity of hNBC3 expressed in HEK293 cells. Fig. 2*A* shows that non-transfected HEK293 cells have small NBC activity, which was largely inhibited by pre-incubating the cells with 0.5 mM DIDS. Fig. 2*C* shows that pre-incubation of acidified cells expressing hNBC3 with 0.5 mM DIDS inhibited Na$^+$-dependent HCO$_3^-$ influx by only $26 \pm 5\%$ ($n = 4$), which can be attributed to the endogenous DIDS-sensitive NBC activity illustrated in Fig. 2*A*.

Interaction between hNBC3 and CFTR—Based on the combined results in Figs. 1 and 2, we can conclude that the expression of the hNBC3 clone in HEK293 cells resulted in the appearance of a DIDS- and EIPA-insensitive Na$^+$-HCO$_3^-$ cotransport activity. In addition, the hNBC3 expressed in HEK293 cells could be detected readily by Western blot using specific anti-hNBC3 antibodies (19). This is illustrated in Fig. 3*A*, *upper panel*. In all of the experiments tested (>10), non-transfected HEK293 cells or HEK293 cells transfected with GFP showed low expression level of hNBC3. The expression of hNBC3 or ΔC-hNBC3 increased the protein level detected by the anti-hNBC3 antibodies ~7-fold. As was found for the activity, there was no discernable difference in the expression of hNBC3 and ΔC-hNBC3. The agreement between protein ex-
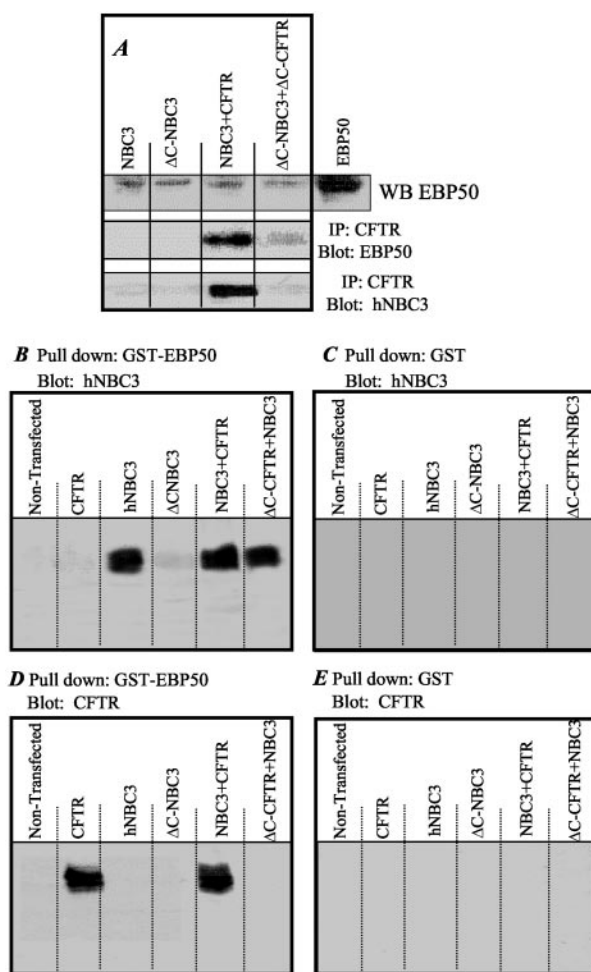

FIG. 4. **Co-IP of EBP50, CFTR, and NBC3 and pull down of CFTR and hNBC3 by GST-EBP50.** *Panel A*, extracts (40 μg of protein) from HEK293 cells transfected with hNBC3, ΔC-hNBC3, hNBC3+CFTR, and ΔC-hNBC3+ΔC-CFTR were used to estimate expression of EBP50 in HEK293 cells (*upper blot*). Cells were also transfected with EBP50 to positively identify the protein (*right-most lane* in *upper blot*). The extracts (400 μg of protein) were also used to immunoprecipitate CFTR (*IP*: *CFTR*) and blot for EBP50 (*Blot*: *EBP50*) and hNBC3 (*Blot*: *hNBC3*). *WB*, Western blot. *Panels B–E*, recombinant GST-EBP50 (*B* and *D*) or GST alone (control) (*C* and *E*) bound to beads was used to pull down CFTR and hNBC3 from 400-μg protein of extracts prepared from non-transfected cells or cells transfected with CFTR, hNBC3, ΔC-hNBC3, ΔC-CFTR, or a combination of the proteins. The *blots* in *B* and *C* were probed for hNBC3, and the blots in *D* and *E* were probed for CFTR.

pression and activity of the hNBC3 clones indicates that indeed the activity measured in Figs. 1 and 2 is attributed to the expressed clones, and the expressed clones can be used to study interaction with CFTR.

As indicated above, CFTR and hNBC3 have the PDZ binding motif in their C terminus. To determine whether the two proteins interact with each other and the importance of the PDZ binding motifs for this interaction, we prepared clones for the expression of CFTR, ΔC-CFTR, hNBC3, and ΔC-hNBC3 and tested the interaction between the proteins by Co-IP. First, we found that the expression of WT-CFTR or ΔC-CFTR did not affect expression of any of the hNBC3 clones (Fig. 3*A*, *upper blot*). We then immunoprecipitated CFTR and probed for hNBC3. Fig. 3*A*, *middle blot*, shows that a similar amount of CFTR was immunoprecipitated from all of the cells. Fig. 3*A*, *bottom blot*, shows that hNBC3 can be coimmunoprecipitated with CFTR and that the deletion of the PDZ binding motifs of CFTR or of hNBC3 markedly reduced Co-IP of hNBC3. A small
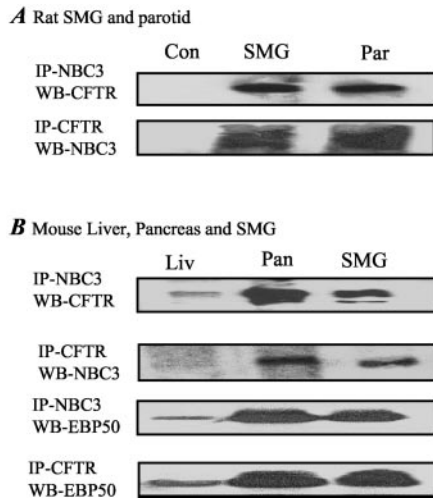
FIG. 5. **Co-IP of CFTR and NBC3 expressed in native tissues.** *A*, extracts were prepared from rat SMG or parotid (*Par*) glands, and 400 μg were used for IP of NBC3 (*IP-NBC3*) and blotting for CFTR (*WB-CFTR*) or IP of CFTR (*IP-CFTR*) and blotting for NBC3 (*WB-NBC3*). For controls (*Con*), SMG extract was used, and the primary antibodies used for the IP were omitted. *B*, Co-IP of CFTR, NBC3, and EBP50 using 400 μg from the mouse liver (*Liv*), pancreas (*Pan*), and SMG. A mixture of primary antibodies and extract from liver (*first lane* in *each blot*) was used as a control. Note that IP of CFTR coimmunoprecipitates NBC3 and EBP50, and IP of NBC3 coimmunoprecipitates CFTR and EBP50.

FIG. 6. **Effect of CFTR on hNBC3 activity.** HEK293 cells stably or transiently expressing hNBC3 or ΔC-hNBC3 were transfected with CFTR or ΔC-CFTR. The cells were loaded with Na^+ by incubation in K^+-free medium containing ouabain, equilibrated in a HCO_3^--buffered medium, and incubated in Na^+-free medium to acidify the cytosol to pH_i of ~6.5. Near the end of the acidification, the cells were stimulated with 5 μM forskolin and treated with 10 μM EIPA while still in Na^+-free medium. In *B*, the cells transfected with hNBC3 and hNBC3+CFTR (*dotted lines*) were also treated with 10 μM H89 after cell acidification. Na^+-HCO_3^- cotransport was then initiated by exposing the cells to a solution containing forskolin, EIPA, and 140 mM Na^+. *A* and *B* show individual examples under each condition, and *C* summarizes the results of 3–9 experiments. Note that the activation of CFTR inhibited hNBC3 activity, which was largely prevented by inhibition of protein kinase A with H89, and that deletion of the PDZ binding motifs of CFTR or hNBC3 prevented the inhibition. NS, not significant.

amount of hNBC3 sometimes coimmunoprecipitated with ΔC-CFTR, and a small amount of ΔC-hNBC3 sometimes coimmunoprecipitated with WT-CFTR. This small amount of Co-IP probably represents nonspecific binding of the NBC3s to the beads because it was not observed in all experiments (see Fig. 4*A*). The reciprocal experiment is shown in Fig. 3*B*. Immunoprecipitation of hNBC3 from cells expressing hNBC3 and WT-CFTR coimmunoprecipitated WT-CFTR. This Co-IP was markedly lower in cells expressing hNBC3 and ΔC-CFTR. Fig. 3*B* shows that a similar amount of CFTR was coimmunoprecipitated by the anti-hNBC3 antibodies from cells expressing only WT-CFTR and cells expressing hNBC3 and ΔC-CFTR.

The results in Fig. 3 clearly show that CFTR interacts with hNBC3 and that the interaction requires the PDZ binding motif of both proteins. Potential scaffolding proteins that mediate the interaction between CFTR and hNBC3 are EBP50 and PDZK1, because both scaffolds bind CFTR (20, 21). Preliminary results showed that HEK293 cells express EBP50; therefore, we tested whether immunoprecipitation (IP) of CFTR can coimmunoprecipitate both hNBC3 and EBP50. The results are shown in Fig. 4*A*. EBP50 was positively identified by expressing the protein in HEK293 cells (Fig. 4*A*, *right lane* in *upper blot*). IP of CFTR coimmunoprecipitated EBP50 and hNBC3, and the Co-IP was abolished when ΔC-hNBC3 was expressed with WT-CFTR. To obtain further evidence for the role of scaffolds like EBP50 in mediating the interaction between the transporters, the proteins were expressed in HEK293 cells and recombinant-purified GST-EBP50 was used for a pull-down assay. Fig. 4, *B* and *C*, show that GST-EBP50 pulled down CFTR and hNBC3 when expressed individually in HEK293 cells (*first two lanes* in *each blot*). Importantly, GST-EBP50 pulled the two proteins when expressed together (*last lane* in *each blot*).

The results in Figs. 3 and 4 suggest that CFTR interacts with hNBC3, and the interaction is mediated by scaffolding proteins with PDZ domains similar to those of EBP50. These results were obtained in expression systems. It is essential to determine whether such interaction between the proteins occurs *in*
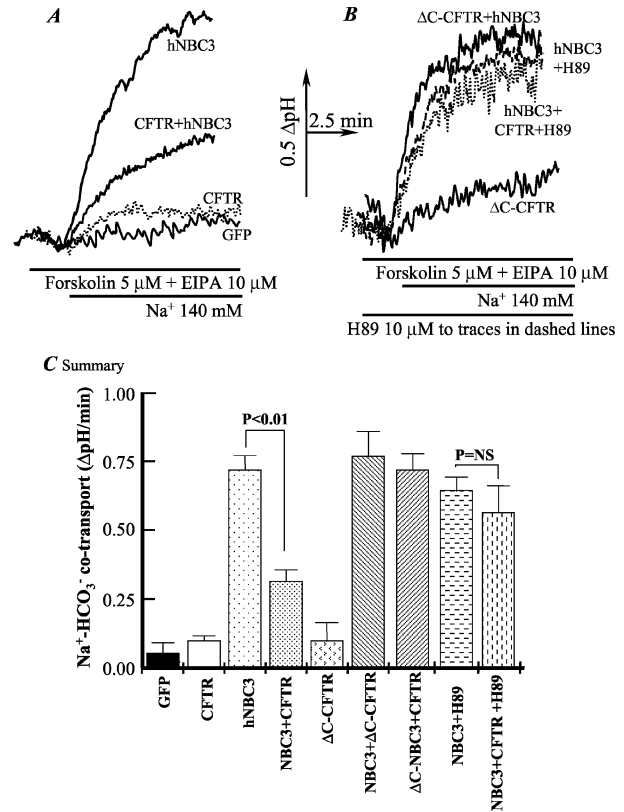
vivo. To test this, we attempted to coimmunoprecipitate CFTR and NBC3 from the rat SMG and parotid glands and the mouse pancreas and SMG. These secretory glands were selected because they express high levels of CFTR (1). The SMG duct and acinar cells express several splice variants of NBCn1 (9), and RT-PCR analysis showed similar expression of NBCn1 splice variants in the pancreas.[2] Fig. 5*A*, *upper blot*, shows that immunoprecipitation of rat NBC3 coimmunoprecipitates CFTR, and Fig. 5*A*, *lower blot*, shows that immunoprecipitation of rat CFTR coimmunoprecipitates NBC3. Similarly, Fig. 5*B* shows the reciprocal Co-IP of the proteins from the mouse pancreas and SMG. Interestingly, immunoprecipitation of CFTR or NBC3 coimmunoprecipitated EBP50 from the pancreas and SMG, indicating that both proteins may interact *in vivo* with the PDZ binding domain(s) of EBP50. Little or no signal was observed with liver extract, probably because CFTR is expressed only in bile ducts and the majority of the membranes are from hepatocytes. At any rate, liver extracts serve as a good negative control for the coimmunoprecipitate observed with pancreatic and SMG extracts.

The interaction between NBC3 and CFTR *in vivo* and in

---

[2] X. Luo and S. Muallem, unpublished observation.

heterologous systems raised the possibility that CFTR regulates NBC3 activity in a manner similar to the regulation of NHE3 activity by CFTR (22). To test this possibility, hNBC3 and ΔC-hNBC3 were coexpressed with CFTR or ΔC-CFTR, and NBC activity was measured. Figs. 6, *A* and *B*, show individual experiments, and Fig. 6*C* summarizes the results of 3–9 experiments under each condition. To compare NBC activity under the same conditions, all cells were loaded with Na^+ and equilibrated in a HCO_3^--buffered medium and then were incubated in a HCO_3^--buffered Na^+-free medium to deplete the cells of Na^+ and acidify the cytosol (including the cytosol of control cells transfected with GFP). When pH_i was close to 6.5, all cells were stimulated with forskolin to activate CFTR and incubated with 10 μM EIPA to inhibit the NBC-independent mechanisms. In separate control experiments, we found that unstimulated CFTR had no effect on hNBC3 activity (data not shown), confirming the Western blot data in Fig. 3*A*, which show that the expression of CFTR had no effect on expression of hNBC3. Na^+-HCO_3^- cotransport activity was initiated by perfusing the cells with medium containing forskolin, EIPA, and 140 mM Na^+. The expression of CFTR or ΔC-CFTR alone had minimal effect on the rate of alkalinization (Fig. 6, *A* and *B*), although CFTR activates Cl^- and HCO_3^- transport (13, 15). This is probably because Cl^- and HCO_3^- transport is very low at pH_i of 6.5 at which internal $[HCO_3^-]$ is very low. However, stimulated WT-CFTR inhibited hNBC3 activity by ~67 ± 11% ($n = 7$, $p <$ 0.01) (Fig. 6, *A* and *C*). Notably, ΔC-CFTR had no effect on hNBC3 activity, and WT-CFTR had no effect on the activity of ΔC-hNBC3 (Figs. 6, *B* and *C*). Finally, to further show that activation of CFTR was required for inhibition of hNBC3, we tested the effect of the protein kinase A inhibitor H89. H89 alone slightly inhibited the activity of hNBC3. The mechanism and significance of this inhibition were not investigated here. Significantly, treatment with H89 prevented inhibition of hNBC3 activity by stimulated CFTR (Fig. 6, *B* and *C*). Hence, activated CFTR inhibits hNBC3 activity, and this inhibition required the interaction among the proteins that is mediated by their PDZ binding motifs.

In summary, in this work, we provide biochemical and functional evidence for regulatory interaction between CFTR and hNBC3. This interaction required the PDZ binding motifs of both proteins, suggesting that the interaction is indirect and is probably mediated by scaffolding protein such as EBP50 or PDZK1. The consequence of this interaction is the ability of activated CFTR to inhibit the activity of hNBC3. Importantly, not only are CFTR and NBC3 expressed in the LM of secretory cells (1, 9, 24), they could be coimmunoprecipitated (Fig. 5), indicating that the two proteins also interact *in vivo*. The regulation of NBC3 activity by CFTR lend further support to the idea that CFTR regulates the entire process of HCO_3^- transport across the LM of secretory epithelial cells. Thus, activated CFTR dramatically activates Cl^- and HCO_3^- transport (11, 15, 23) and at the same time inhibits HCO_3^- salvage by NHE3 (13) and NBC3 (this work). Two obvious questions are how CFTR can regulate so many transporters and transport functions and how EBP50, which has only two PDZ domains, can mediate so many interactions? CFTR is probably a central member of a protein complex in the luminal membrane of secretory epithelial cells. The scaffolding protein(s) assembling the complex is not known with certainty. Secretory cells express more than one scaffolding protein in their luminal pole that can bind CFTR. Furthermore, the complex probably contains more than one scaffolding protein, similar to other multi-proteins complexes as found in the postsynaptic density (24) and in caveolae (25). Thus, at present, it is not clear how the HCO_3^- transport complex is assembled and whether CFTR and

NBC3 bind to the same scaffold. Nonetheless, their ability to bind to PDZ binding domains is one mechanism by which CFTR can function as a HCO_3^- sensor governing the activity of the HCO_3^--transporting complex.

Interestingly, in a previous work (9), we showed that both SMG acinar and duct cells express NBC3, although the two cell types express different splice variants of the protein. In addition, localization of NBC3 isoforms is cell-specific. NBC3 splice variants were localized to the luminal membrane of SMG cells (9) intercalated cells, and the cortical-collecting duct (26–28) but to the basolateral membrane of the thick ascending limb (25) and duodenal enterocytes (29). If the activity of NBC3 is regulated in all cells and in all membranes by regulatory interaction with other proteins, it is possible that other ABC transporters or proteins that interact with the scaffold(s), which binds NBC3, can regulate the activity of NBC3 in a manner similar to that of CFTR described here. Hence, it will be of interest in the future to examine the regulation of NBC3 and other HCO_3^- transporters with PDZ binding motifs by members of the ABC transporters family. Such a regulation may be a general mechanism for the regulation of cellular HCO_3^- transport.

The physiological significance of the findings in this work remains to be fully established because of the insensitivity of hNBC3 activity to inhibition by EIPA. Although NBC3 is expressed in the luminal membrane of secretory ducts (9), the activity found in the duct is DIDS-insensitive but EIPA-sensitive. However, the cells express more than one splice variant of NBC3. This finding raises the possibility that the splice variants interact with each other to mediate NBC3 activity. Thus, it is possible that substrate specificity and sensitivity to blockers are functions of the exact splice variants expressed in a cell type and the interaction between them. Indeed, SMG acinar and duct cells express different NBC3 splice variants. The transporters in acinar cells transport HCO_3^- but not OH^-, whereas the transporters in the duct transport both HCO_3^- and OH^-. An analysis of the behavior of all splices variants and the relationship between them is required to fully address this problem. Nevertheless (*a*) the expression of NBC3 and CFTR in the luminal membrane HCO_3^--transporting complex, their PDZ domain-mediated interaction, and inhibition of NBC3 activity by stimulated CFTR suggests that NBC3 plays a major role in HCO_3^- salvage. In this manner, CFTR regulates this process by regulating the activity of both NHE3 and NBC3.

REFERENCES

1. Zeng, W., Lee, M. G., Yan, M., Diaz, J., Benjamin, I., Marino, C. R., Kopito, R., Freedman, S., Cotton, C., Muallem, S., and Thomas, P. (1997) *Am. J. Physiol.* **273,** C442–C455
2. Tizzano, E. F., and Buchwald, M. (1995) *Ann. Intern. Med.* **123,** 305–308
3. Quinton, P. M. (1999) *Physiol. Rev.* **79,** S3–S22
4. Grubb, B. R., and Boucher, R.C. (1999) *Physiol. Rev.* **79,** S193–S213
5. Pilewski, J. M., and Frizzell, R. A. (1999) *Physiol. Rev.* **79,** S215–S255
6. Zhao, H., Star, R. A., and Muallem, S. (1994) *J. Gen. Physiol.* **104,** 57–85
7. Ishiguro, H., Steward, M. C., Lindsay, A. R., and Case, R. M. (1996) *J. Physiol.* **495,** 169–178
8. Abuladze, N., Lee, I., Newman, D., Hwang, J., Boorer, K., Pushkin, A., and Kurtz, I. (1998) *J. Biol. Chem.* **273,** 17689–17695
9. Luo, X., Choi, J. Y., Ko, S. B., Pushkin, A., Kurtz, I., Ahn, W., Lee, M. G., and Muallem, S. (2001) *J. Biol. Chem.* **276,** 9808–9816
10. Argent, B. E., and Case, R. M. (1994) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed) 3rd Ed., pp. 1478–1498, Raven Press, Ltd., New York
11. Lee, M. G., Choi, J. Y., Luo, X., Strickland E., Thomas, P. J., and Muallem, S. (1999) *J. Biol. Chem.* **274,** 14670–14677
12. Kopelman, H., Corey, M., Gaskin, K., Durie, P., Weizman, Z., and Forstner, G. (1988) *Gastroenterology* **95,** 349–355
13. Lee, M. G., Ahn, W., Choi, J. Y., Luo, X., Seo, J. T., Schultheis, P. J., Shull, G. E. Kim, K. H., and Muallem, S. (2000) *J. Clin. Invest.* **105,** 1651–1658
14. Schultheis, P. J., Clarke, L. L., Meneton, P., Miller, M. L., Soleimani, M., Gawenis, L. R., Riddle, T. M., Duffy, J. J., Doetschman, T., Wang, T.,

Giebisch, G., Aronson, P. S., Lorenz, J. N., and Shull, G. E. (1998) *Nat. Genet.* **19,** 282–285

15. Choi, J. Y., Muallem, D., Kiselyov, K., Lee, M. G., Thomas, P. J., and Muallem, S. (2001) *Nature* **410,** 94–97
16. Wang, S., Raab, R. W., Schatz, P. J., Guggino, W. B., and Li, M. (1998) *FEBS Lett.* **427,** 103–108
17. Shenolikar S., and Weinman E. J. (2001) *Am. J. Physiol.* **280,** F389–F395
18. Choi, I., Aalkjaer, C., Boulpaep, E. L., and Boron, W. F. (2000) *Nature* **405,** 571–575
19. Pushkin, A., Abuladze, N., Lee, I., Newman, D., Hwang, J., and Kurtz, I. (1999) *J. Biol. Chem.* **274,** 16569–16575
20. Short, D. B., Trotter, K. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R. C., Stutts, M. J., and Milgram, S. L. (1998) *J. Biol. Chem.* **273,** 19797–19801
21. Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) *Cell* **103,** 169–179
22. Ahn, W., Kim, K. W., Lee, J. A., Kim, J. Y., Choi, J. Y., Moe, O. M., Milgram, S. L., Muallem, S., and Lee, M. G. (2001) *J. Biol. Chem.* **276,** 17236–17243
23. Lee, M. G., Wigley, W. C., Zeng, W., Noel, L. E., Marino, C. R., Thomas, P. J., and Muallem, S. (1999) *J. Biol. Chem.* **274,** 3414–3421
24. Blackstone, C., and Sheng, M. (2002) *Front. Biosci.* **7,** 872–885
25. Anderson, R. G., and Jacobson, K., (2002) *Science* **296,** 1821–1825
26. Kwon, T. H., Fulton, C., Wang, W., Kurtz, I., Frokiaer, J., Aalkjaer, C., and Nielsen, S. (2002) *Am. J. Physiol.* **282,** F341–F351
27. Pushkin, A., Yip, K. P., Clark, I., Abuladze, N., Kwon, T. H., Tsuruoka, S., Schwartz, G. J., Nielsen, S., and Kurtz, I. (1999) *Am. J. Physiol.* **277,** F974–F981
28. Pushkin, A., Clark, I., Kwon, T. H., Nielsen, S., and Kurtz, I. (2000) *J. Androl.* **21,** 708–720
29. Praetorius, J., Hager, H., Nielsen, S., Aalkjaer, C., Friis, U. G., Ainsworth, M. A., and Johansen, T. (2001) *Am. J. Physiol.* **280,** G332–G343

**The Cystic Fibrosis Transmembrane
Conductance Regulator Interacts with and
Regulates the Activity of the HCO Salvage
Transporter Human Na $^+$-HCO
Cotransport Isoform 3**

Meeyoung Park, Shigeru B. H. Ko, Joo Young
Choi, Gaia Muallem, Philip J. Thomas,
Alexander Pushkin, Myeong-Sok Lee, Joo
Young Kim, Min Goo Lee, Shmuel Muallem
and Ira Kurtz