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Rab4GTPase modulates CFTR function by impairing channel expression at plasma membrane

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Abstract

Cystic fibrosis (CF), an autosomal recessive disorder, is caused by the disruption of biosynthesis or the function of a membrane cAMP-activated chloride channel, CFTR. CFTR regulatory mechanisms include recruitment of channel proteins to the cell surface from intracellular pools and by protein–protein interactions. Rab proteins are small GTPases involved in regulated trafficking controlling vesicle docking and fusion. Rab4 controls recycling events from endosome to the plasma membrane, fusion, and degradation. The colorectal cell line HT-29 natively expresses CFTR and responds to cAMP stimulation with an increase in CFTR-mediated currents. Rab4 overexpression in HT-29 cells inhibits both basal and cAMP-stimulated CFTR-mediated currents. GTPase-deficient Rab4Q67L and GDP locked Rab4S22N both inhibit channel activity, which appears characteristically different. Active status of Rab4 was confirmed by GTP overlay assay, while its expression was verified by Western blotting. The pull-down and immunoprecipitation experiments suggest that Rab4 physically interacts with CFTR through protein–protein interaction. Biotinylation with cell impermeant NHS-Sulfo-SS-Biotin implies that Rab4 impairs CFTR expression at cell surface. The enhanced cytosolic CFTR indicates that Rab4 expression restrains CFTR appearance at the cell membrane. The study suggests that Rab4 regulates the channel through multiple mechanisms that include protein-protein interaction, GTP/GDP exchange, and channel protein trafficking. We propose that Rab4 is a dynamic molecule with a significant role in CFTR function.

Keywords: CFTR; Rab4; Regulation; GTP/GDP exchange; Trafficking

Cystic fibrosis (CF), an autosomal recessive disorder most common in the Caucasian population, is caused by the disruption of biosynthesis or the function of a membrane cAMP-activated chloride channel, the CF transmembrane conductance regulator (CFTR) [1,2]. The channel mediates electrolyte transport across the luminal surfaces of a variety of epithelial cells. The predominant mutation is the loss of phenylalanine at position 508 that culminates into a misprocessed and mislocalized channel in the cytoplasm, unable to reach its appropriate location as a cAMP-regulated channel in the cell membrane [3,4]. On the other hand, the excessive CFTR activity results in toxin-induced secretory diarrhea [5,6]. CFTR is expressed in a variety of tissues and cell systems, including lung microvasculature [7–9] and colon [10]. CFTR plays a predominant role in both cAMP- and Ca^{2+}-activated secretion of electrolytes. The extent to which CFTR Cl^- channels are regulated by protein–protein interactions is largely unknown. CFTR has been shown to interact with two components of soluble *n*-ethylmaleimide-sensitive attachment receptor (SNARE) machinery that mediates membrane fusion reactions.

Ras-related Rab GTPases regulate intracellular trafficking and secretions [11,12]. These small 21–25 kDa proteins are activated by cycling between GTP-active and GDP inactive states, and are localized in specific compartments [13–15]. Rab proteins and their effectors act upstream of SNAREs to regulate the initial stages of membrane tethering and fusion [16,17]. The regulatory principles of Rab proteins and other GTPases reside in their ability to switch between GTP- and GDP-bound conformations [18–22].

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Rabs cycle between membrane-bound and a cytosolic state [23], and are regulated by various accessory factors [24]. In resting cells, Rab GTPases exist mostly in GDP-bound form and in complexes with Rab GDI in the cytosol [25]. The GTP-bound form of Rab GTPases is associated with cell membranes. Rab4 presence has been demonstrated in early and late endosomal compartment recycling and recycling to the plasma membrane. Alternatively, Rab4GTPase can also effect the degradative endosomal trafficking pathways [26]. Recently, we defined the regulation of epithelial sodium channel by Rab isoforms [27,28].

In the present communication, we demonstrate that over-expression of Rab4 in HT-29 colonic epithelial cell line inhibits CFTR chloride channel activity. Our data further emphasize that Rab4 inhibition of CFTR activity is associated with the GTP/GDP exchange mechanism, which impairs CFTR cell membrane expression.

Methods

Materials and reagents. HT-29 cells were purchased from American type culture collection (ATCC # HTB-38) (Manassas, VA). The anti-Rab4 mouse monoclonal antibody, and other antibodies and reagents were obtained from Abcam (Cambridge, MA). Rab4 clones [29] were a kind gift from Dr. Hiroshi Shibata, Gunma University, Japan. The CFTR antibodies were available from Chemicon, (Temecula, CA), and Santa Cruz Biotechnology, (Santa Cruz, CA). Lipofectamine was purchased from Invitrogen Life Technologies (Carlsbad, CA). Horseradish peroxidase-conjugated (HRP-conjugated) secondary antibodies (anti-rabbit and anti-mouse) were from Pierce Chemical (Rockford, IL). Chariot protein delivery system was available from Active Motif (Carlsbad, CA). RIPA buffer contained 50 mM Tris–Cl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, and 0.2% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM EDTA with protease inhibitors. Ni–NTA-beads for the purification of His-tagged proteins were purchased from Qiagen (Valencia, CA). All other reagents, if not mentioned, were purchased from Sigma Chemical Laboratory (St Louis, MO).

Cell line and measurements of Short Circuit Current (Isc). HT-29 cells were cultured in McCoy's 5a medium with 1.5 mM L-glutamine and 10% fetal bovine serum in 5% CO_2 at 37 °C. The cells were grown on Falcon12 or 24-well inserts for all the experiments and maintained to determine the cAMP-dependent CFTR-mediated currents of the Isc [30,31]. CFTR-mediated currents were recorded in two ways. Confluent monolayers were mounted in a modified Ussing chamber (Trans-24 miniperfusion chamber, Warner Instruments, Hamden, CT). Apical and basolateral chambers were continuously bathed with medium and Isc were measured with transepithelial voltage clamped at 0 mV with a DVC-1000 dual voltage clamp. Voltage pulses (10 mV) were applied every 3 min to monitor the transepithelial resistance. cAMP-dependent activation was achieved by adding cocktail containing IBMX (100 μM), dibutyryl-adenosine-cAMP (200 μM), and forskolin (20 μM) for 15 min. After the initial measurements, 1 mM diphenylamine carboxylate (DPC) was added, and CFTR chloride currents were expressed as the DPC-sensitive component of the Isc. Alternatively, the Isc were also recorded with EVOM epithelial voltohmeter using STX2 electrode (WPI, Sarasota, FL).

GTP overlay assay. HT-29 transfected with Rab4 constructs were solubilized in SDS sample buffer. Samples (15 μg of protein) from untreated HT-29 cells; or transfected with Rab4WT, Rab4S22N, and Rab4Q67L were resolved on a 12% SDS–PAGE and transferred to nitrocellulose filters. The blot was incubated for 60 min in binding buffer containing 32 P-labelled GTP and exposed to X-ray film. To define Rab4 expression, the GTP blot was then stripped of nucleotide and developed using a monoclonal anti-Rab4 serum. Bands were visualized after incubation with anti-mouse HRP-labeled secondary antibody and ECL.

CFTR pull-down assays. Soluble eluted His-Rab4 proteins were added to a Triton X-100 lysate (1% Triton X-100 in PBS plus 1 mM phenylmethanesulfonyl fluoride and 1 μg/ml each of leupeptin, pepstatin, and aprotinin) of cells. After diluting the samples in PBS to bring the final Triton concentration to 0.2%, the samples were mixed for 3–12 h at 4 °C. The bound proteins were then precipitated with excess Ni–NTA–agarose, washed extensively according to manufacturer's instructions, and analyzed for CFTR by immunoblotting using monoclonal antibody (Chemicon). Duplicate lysate samples were assayed for CFTR protein amount by immunoprecipitation followed by immunoblotting using the same antibody. The amount of CFTR was quantitated by densitometry and analyzed by using IP Lab software (Signal Analytics, Vienna, VA).

Cell surface biotinylation and CFTR detection. Forty-eight hours after transfection, HT-29 were incubated in 5 ml of Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL) (0.5 mg/ml) in borate buffer at 4 °C for 30 min. After washing three times with ice-cold quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3), cells were solubilized on ice in 500 μl of immunoprecipitation buffer (1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 10 mM Tris–Cl, pH 7.5) containing a cocktail of protease inhibitors (Sigma). The cell lysate was centrifuged for 20 min at 16,000*g*, and the supernatant was retained. Fifty microliters of immobilized streptavidin resin (Pierce) (50% slurry in phosphate-buffered saline containing 2 mM NaN_3) was added to the supernatant, which was then incubated overnight at 4 °C with gentle rocking. Samples were centrifuged for 2 min at 8000*g*, and the resin was washed five times with immunoprecipitaton buffer. The protein was eluted from the resin by the addition of SDS–PAGE sample buffer containing 5% 2-mercaptoethanol and incubation at 65 °C for 5 min. The samples were analyzed for CFTR expression by Western blot analysis and detected with monoclonal antibody. The blots were raised using enhanced chemiluminescence (ECL) and the films were developed using autoradiography.

Immunoprecipitation experiments. Cells were solubilized in RIPA buffer and washed. The supernatant recovered after 14,000 rpm centrifugation for 15 min was used for immunoprecipitation. The lysates were precleared by incubating with protein A–Sepharose beads for 1 h at 4 °C and centrifugation. The supernatants were further incubated with a specific antibody for 2 h at 4 °C, followed by incubation with Sepharose beads (Sigma) for 2 h at 4 °C. After washing with RIPA buffer beads were solubilized in SDS sample buffer and run on SDS–PAGE. The proteins were transferred to PVDF membrane. Bound antibody was detected by enhanced chemiluminescence and quantitated by phosphor-imaging under conditions where there was a linear relationship between intensity and pixel number. In some cases, membranes were stripped of bound antibodies and re-probed with additional antibodies. Following the manufacturer's instructions, membranes were immersed in stripping buffer (62.5 mM Tris–HCl (pH 6.7), 2% SDS, and 100 mM mercaptoethanol) for 30 min at 55 °C, and then washed extensively in Tris-buffered saline (0.1 M, pH 7.4, 0.05% Tween 20) at room temperature. Membranes were re-blocked in milk-TBS, incubated with the desired antibody, and raised for protein detection using ECL as described above.

Electrophoresis, immunoblotting, and characterization of proteins. The proteins were solubilized at 70 °C for 15 min in Laemmli sample buffer and run through SDS–PAGE in 10% polyacrylamide gels. The proteins were transferred electrophoretically to the PVDF membrane. After blocking with 5% non-fat dried milk in TBS–Tween (Tris-buffered saline, 0.05% Tween 20, pH 7.4), the membranes were probed with polyclonal or monospecific affinity-purified anti-peptide antibodies. Blots were developed using ECL and visualized by light-sensitive imaging film (Kodak). Quantification was carried out with densitometry.

Cytosolic protein preparation. The cytosolic or internal pools of proteins were isolated the following way. The cells were suspended in the extraction buffer (50 mM Tris, pH 7.3, 250 mM sucrose, 2 mM EDTA, 1% Triton X-100, and 2 mM protease inhibitor), vortexed vigorously, and solubilized in Dounce homogenizer. The lysates were centrifuged at 2800*g* at 4 °C for 20 min and the supernatants were collected. The debris from above preparations was resuspended in extraction buffer and supernatants were recollected as described above. Finally, ice-cold aqueous 0.1 M

Na_2CO_3 solution was added to the pooled supernatants and agitated gently on ice at 4 °C for 1 h. The preparation was ultracentrifuged at 100,000*g* for 1 h and the supernatant was used to assay CFTR internal pools. The CFTR detection was done by Western blot analysis using monoclonal antibody.

Delivery of antibodies. The HT-29 cells were targeted with anti-Rab4, or anti-CFTR or irrelevant IgG (anti-GST) using the Chariot protein delivery system. Antibodies were complexed with the Chariot reagent at a ratio of 1 ng IgG: 2μl Chariot in 100 μl PBS for 30 min. Then the IgG: Chariot complexes were overlaid onto cultured cells in the presence of fresh culture medium for 3 h, and basal and cAMP currents were measured as described before.

Statistical analysis. A paired test or analysis of variance for multiple comparisons was used for statistical analysis. A p value less than 0.05 was considered significant.

Results

Our preliminary studies established that cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in colonic epithelial HT-29 cells. We used two techniques: (a) RT-PCR and (b) Western blot analysis to confirm our findings (data not shown). Previous studies also support our findings [32–34]. We also determined if these cells respond to cAMP stimulation. Our observations suggested a strong stimulation of basal currents with cAMP cocktail containing IBMX (100 μM), dibutyryl-adenosine-cAMP (200 μM), and forskolin (20 μM) (Fig. 1), and is comparable with the observed findings of other investigators.

In order to define if Rab proteins can modulate CFTR function, we transiently transfected HT-29 cells with Rab isoforms (1a, 3, and 4) and recorded CFTR currents in basal or cAMP induced cells 48 h post-transfection. We did not observe any discernible effect of control vector or Rab1a and Rab3 transfection of either basal or CFTR-mediated currents (data not shown), however, Rab4 over-expression resulted in considerable modulation of both basal and CFTR-mediated currents in these colonic epithelial HT-29 cells. Therefore, we focused our efforts in analyzing the effect of this Rab isoform. For this, we transfected HT-29 cells with Rab4, an isoform implicated in endosomal recycling and plasma membrane trafficking, and two of its mutants GTPase-deficient Q67L and GDP-locked S22N, and recorded the basal and cAMP-stimulated currents (Fig. 1). Wild-type Rab4 blocked both basal and cAMP-induced currents. Over-expression of GTP-locked Rab4, however, could not impose more inhibition of cAMP-induced currents than the wild-type Rab4. On the other hand, S22N (GDP status) maximally blocked both basal and cAMP-induced CFTR currents. Most of these currents were inhibited almost immediately by applying 1 mM DPC to the bath solution. We characterized and confirmed the expression of each transfection by Western blot analysis (Fig. 2). Moreover, we confirmed the intensity of each expression by densitometry. Our analysis also confirmed the mild expression of native Rab4 in HT-29 cells.

The characterization of the Rab4 dominant negative (S22N) and constitutively activated (Q67L) mutants were verified by their biochemical behavior. The lysates from HT-29 cells expressing Rab4 constructs were analyzed by SDS–PAGE, GTP overlay assay, and immunoblotting (Fig. 2). The Rab4S22N mutant was deficient in GTP binding, while wild-type and constitutively activated Rab4 efficiently bound GTP. In order to confirm equivalent sample loading and expression of all Rab4 constructs, the GTP blot was stripped of nucleotide, stripping verified by autoradiography, and developed as an immunoblot with a monoclonal anti-Rab4 serum. All Rab4 constructs are expressed at similar levels and vastly in excess of the endogenous protein. We also observed mild endogenous Rab4 in the immunoblot. However, a strong signal is obtained for all Rab4 constructs. These data confirm the active status

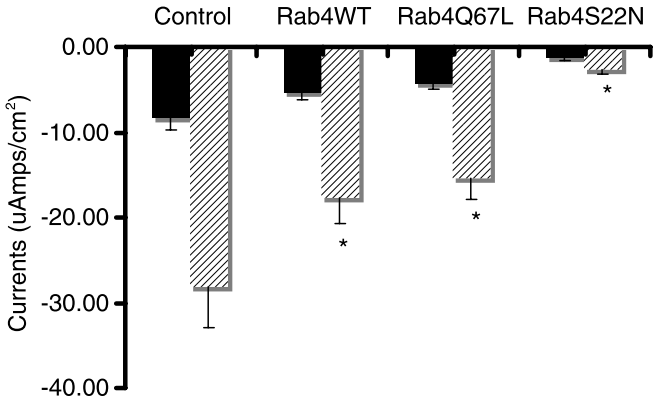

Fig. 1. Rab4 inhibits CFTR-mediated currents. HT-29 cells grown on cell inserts were transfected with wild-type Rab4 and its mutants. Forty-eight hours later, the basal (closed bars) and cAMP induced currents (hatched bars) were recorded. The data represent means of three individual experiments. The currents could be inhibited by the addition of 1 mM diphenylamine 2-carboxylate (DPC) to the bath solution. Conditions that resulted in a significant change ($p < 0.05$) from the control (panel 1—closed bar) are denoted with an asterisk.

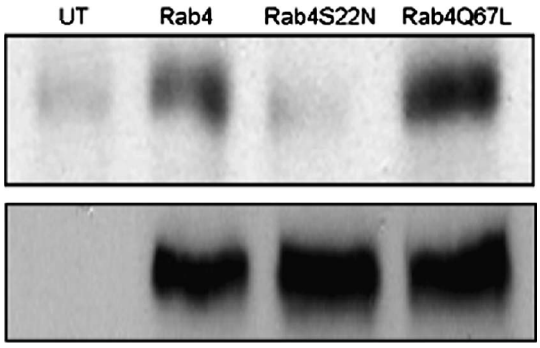

Fig. 2. GTP overlay assay (top panel) of HT-29 cells transfected with Rab4WT and mutants. HT-29 cells were transfected with His-Rab4 constructs and lysed 48 h later. Samples (15 g of protein) from untreated UT (lane 1), or transfected Rab4WT (lane 2), Rab4S22N (lane 3), and Rab4Q67L (lane 4), were resolved on a 12% SDS–PAGE and transferred to nitrocellulose filters. The blot was incubated for 60 min in binding buffer containing ^{32}P-labeled GTP and exposed to X-ray film (bottom). Immunoblot analysis: the GTP blot was then stripped of nucleotide and developed using a mouse anti-Rab4 monoclonal serum. Bands were visualized after incubation with anti-mouse HRP-labeled secondary antibody and ECL.

of Rab4 and also point to the role of GTP/GDP exchange in regulating CFTR function.

In order to confirm the inhibitory effect of Rab4, we blocked the expression of native proteins by delivering the specific antibody with Chariot [35,36] system (Fig. 3). The CFTR antibody was used as the control, which completely abrogated CFTR Cl- currents in HT-29 cells on the expected lines while the Rab4 antibody documented stimulatory currents. At the same time, the introduction of Rab3 or syntaxin4 (hatched bars, Fig. 3) antibodies failed to compromise CFTR-mediated currents confirming their non-involvement with CFTR but asserting the specificity of Rab4–CFTR interaction in HT-29 cells. These results suggest that Rab4 (a) inhibits endogenous CFTR in HT-29 cells and (b) that Rab4 plays a physiologically significant role in CFTR regulation.

The expression of cDNA constructs was confirmed by Western blot analysis with specific antibody. Our next goal was to know if the regulatory effect was due to protein–protein interaction between Rab4 and CFTR. In order to achieve this goal, we used His-tagged Rab4 constructs to pull down CFTR from the HT-29 cell lysate. The pulled proteins were fractionated through SDS–PAGE and transferred by wet transfer to a PVDF membrane. The blots were probed with affinity-purified polyclonal or monoclonal CFTR antibodies. We observed detectable bands with the CFTR antibody (Fig. 4). These bands were eliminated by pre-incubating the antibody with concomitant epitope peptide, thus confirming the specificity of interaction between these two proteins. This interaction was reconfirmed by immunoprecipitating HT-29 cell lysates with wild-type Rab4.

In order to evaluate if diminished CFTR activity might be due to changes in CFTR apical expression, we biotinylated the Rab4-transfected cells with cell impermeant non-reducible NHS-Sulfo-SS-Biotin at 4 °C. The biotinylated proteins were pulled-down with streptavidin–agarose beads, solubilized, and separated on SDS–PAGE. The fractionated proteins were transferred to PVDF membrane and probed with CFTR antibody. We observed a formidable reduction in CFTR expressed at the apical membrane in Rab4 transfected cells (Fig. 5), suggesting that CFTR pools are either retained intracellularly by Rab4 or Rab4 interferes with CFTR translocation to the plasma membrane. We further characterized if the changes in the CFTR expression were the consequence of diminished steady-state density of the total cellular CFTR pool. In order to accomplish this goal, we prepared whole cell lysates from HT-29 cells transfected with Rab4 clones by RIPA lysis, loaded equal amounts of proteins on SDS–PAGE, and detected CFTR by Western blot analysis (Fig. 5; bottom panel). Our data indicate that total cellular CFTR does not change and thus supports the hypothesis that Rab4 impairs CFTR expression at the cell surface by retaining the channel in cytosol. In order to confirm this, we isolated the cytosolic fraction of cellular lysates as described in Methods. This

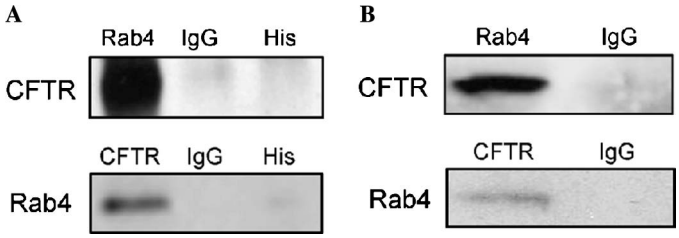

Fig. 4. CFTR interacts with Rab4. Pull-down assay: (A) His-Rab proteins or CFTR antibody was used to pull-down CFTR or Rab4 from the HT-29 cells. The pulled proteins were solubilized and fractionated by SDS–PAGE, transferred to PVDF membrane, and probed with Rab4 or CFTR antibodies. His or IgG were used as control. (B) Immunoprecipitation using anti-Rab4 (top) or anti-CFTR (bottom) antibody. The blots were probed with CFTR or Rab4 antibodies, respectively. The blots were probed with CFTR or Rab4 antibody to confirm the interaction.

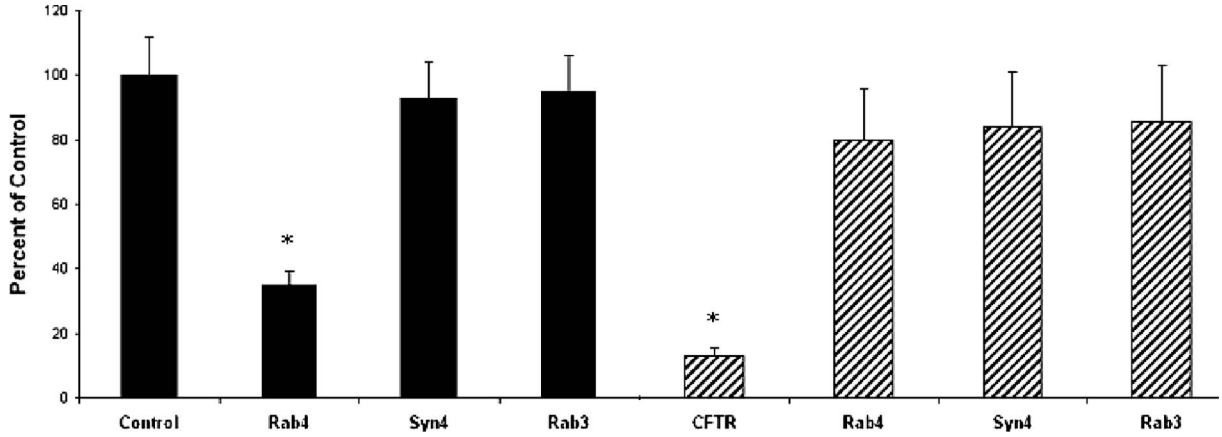

Fig. 3. Anti-Rab4 antibody reverses inhibition of CFTR-mediated currents. HT-29 cells grown on cell inserts were transfected with wild-type Rab4, Syn4, and Rab3 constructs, respectively (closed bars). CFTR-mediated currents were recorded as cAMP-induced and DPC-inhibited currents. CFTR-mediated currents were also recorded in the presence of indicated antibodies (hatched bars) by using the Chariot delivery system. CFTR monoclonal antibody (panel 5—hatched bar) was used as a positive control. The introduction of Rab4 antibody (panel 6—hatched bar) recuperates channel activity observed with Rab4 transfection (panel 2—closed bar). Syn4 or Rab3 antibodies failed to revert Rab4 inhibition (panels 7 and 8—hatched bars). These data show that Rab4 specifically inhibits CFTR-mediated currents in HT-29 cells. The data represent means of three individual experiments. Conditions that resulted in a significant change ($p < 0.05$) from the control (panel 1—closed bar) are denoted with an asterisk.

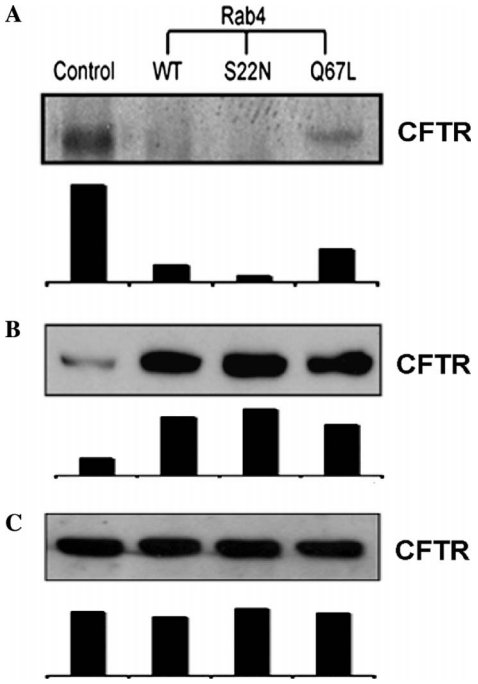

Fig. 5. Rab4 decreases cell surface expression of CFTR while increasing the cytosolic pool. (A—top panel) HT-29 cells grown on cell inserts were transfected with wild-type Rab4 and Rab4Q67L and Rab4S22N. Cell surface proteins were biotinylated with NHS-SS-Biotin, pulled-down with streptavidin–agarose, separated by SDS–PAGE, and transferred to PVDF membrane. All blots described in this figure were probed with CFTR polyclonal antibody. Intracellular cytosolic proteins were recovered as described in the text (B—middle panel-top). Data show that Rab4 increases cytosolic CFTR. (C—bottom panel-top). Total cellular proteins were prepared from by RIPA lysis. An equal amount of cellular proteins was separated by SDS–PAGE and transferred to PVDF membrane. Data reflect no change in total cellular CFTR pool. Protein density was analyzed for each blot by phosphorimaging system (bottom panel of A–C).

preparation upon analysis revealed that in Rab4 expressing cells, a large amount of cellular CFTR is localized internally (Fig. 5; middle panel) and thus possibly does not contribute to the measured channel function.

Discussion

Cystic fibrosis transconductance regulator (CFTR) is a specialized channel, which not only belongs to the ATP-binding cassette (ABC) transporter but also interacts with and regulates a variety of ion channels and transporters. This cAMP-activated channel is expressed on the apical side of epithelial cells and its most prominent mutant Δ508, a causative agent of cystic fibrosis, is deficient in plasma membrane appearance. Hence, CFTR transport to the plasma membrane from intracellular compartments assumes significance. Using the HT-29 colonic epithelial cell system, we have specified that the over-expression of Rab4 modulates CFTR function in these cells. At first, we confirmed the expression of CFTR with cAMP inducible currents in HT-29 cells, which confirms the previous report of CFTR expression in both the proximal and distal colon [37–40] and HT-29 cells [32,34,41,42].

The observations suggest that Rab4 is a critical binding partner and a regulator of CFTR considering its primary role in trafficking, membrane fusion, and recycling, which is supported by our observations in HT-29 cells. Our data suggest that Rab4 regulates CFTR activity through several mechanisms that include: (a) protein–protein interactions, (b) GTP/GDP exchange, and (c) protein trafficking. It is highly likely that the mechanism of Rab4 regulation is complex and might include other pathways. Our observations provide a concrete and formidable proof that Rab4 modulates CFTR function in the cell system, which natively expresses functional CFTR and responds to cAMP stimulation.

Then, we showed that the expression of Rab4 (wild-type) inhibits DPC inhibitable CFTR-mediated currents. Data also suggested that the inhibition of CFTR by Rab4 depends on its GTP-active or GDP-inactive form. Diphenylamine-2-carboxylate (DPC) decreases Cl^- conductance in epithelia and cells of several tissues [43,44], an effect also reported for Na^+ and Ca^{2+} currents, and is equally potent, or even more potent, than that reported for Cl^- channels. It is likely that Rab4 interacts with other DPC responsive channels in this cell line. Therefore, we blocked outwardly rectifying Cl^- currents by DIDS [45,46] and sodium channel currents by using 1 μM amiloride [47,48] and re-recorded the currents. Our observations suggested an almost similar pattern described before suggesting that Rab4 inhibits CFTR channel function. The channel inhibition with both GTP and GDP locked mutants further suggests that Rab4 inhibits channel activity by another mechanism that might include PI3 kinase [49,50] or MAP kinase pathway [51]. Moreover, by delivering anti-Rab4 by the Chariot expression system into HT-29 cells (Fig. 3), we confirmed that Rab4 inhibits CFTR activity in native cells as these cells endogenously express Rab4.

We further characterized the status of expressed Rab4 by GTP overlay assays confirming that its mutants are expressed in correct forms. We also established that Rab4 interacts with CFTR in HT-29 cells. Towards this, we used two measures: (a) by pull-down assay using His-tagged Rab4 proteins (Fig. 4) and (b) by utilizing antibodies against natively expressed Rab4 or CFTR in immunoprecipitation assay (Fig. 4). Our data further suggests that inhibition of CFTR activity might be correlated to the decreased CFTR expression at the cell membrane as confirmed by the biotinylation studies in which we used cell impermeant NHS-Sulfo-SS-Biotin to label and pull-down the apically expressed proteins by streptavidin–agarose in accordance with their specialized localization and function in endosomal recycling and transport [52,53]. Moreover, our data suggest that though Rab4 expression does not alter the steady-state level of cellular CFTR (Fig. 5), it results in the accumulation of CFTR in the intracellular pools (Fig. 5). These observations suggest: (a) that Rab4 is involved in CFTR recycling or its transport to the

plasma membrane and (b) Rab4 effects CFTR function by GTP/GDP exchange or recycling rather than its stabilization in either conformation.

These results were confirmed by employing a battery of antibodies available against both CFTR and Rab4 proteins from two commercial sources (Chemicon and Santa Cruz). These observations support the notion that Rab4 probably restrains the CFTR channel in the intracellular compartments, which results in its reduced expression on the cell membrane critical for channel function in epithelial cells. Studies in the epithelial cell system on Rab4 are limited. Madin–Darby canine kidney cells transfected with Rab4, the GTP-positive mutant Rab4Q67L, and the GDP-locked mutant Rab4S22N, showed the Rab4 and transferrin receptor distribution from the basolateral to the apical plasma membrane in cells [54]. ClC-5 chloride channels in rat intestinal epithelial cells [55] and peripherally located CFTR in rat mandibular cells [56] both appear to colocalize with Rab4 in recycling endosomes. Therefore, it is likely that the CFTR-Rab4 pool is localized internally in the cytoplasm.

The Rab4 protein has been found to be associated with an early endosomal compartment [53,57], recycling [58,59] and degradation [26]. The expression of both Rab4 mutants Rab4S22N dominant negative mutant (locked in the GDP-bound form) and dominant positive Rab4Q67L (GTPase deficient) strongly inhibits CFTR-mediated currents, suggesting that Rab4 exerts its effect through GDP/GTP exchange rather than its active/inactive status. Moreover, observations also throw a possibility that Rab4 might be important for sorting of CFTR from the early endosomal compartment to the plasma membrane and possibly also the degradative routes, as per its defined role. It remains to be seen if GTP/GDP exchange or the GTP/GDP status determines the net regulatory effect of Rab4 on CFTR function. We speculate that the Rab4 protein performs an important role in the normal functioning of CFTR possibly controlling the formation of membrane buds destined for transport to both recycling and degradative acceptor compartments. It will be unwise not to speculate that Rab4 might act to stimulate CFTR function under different physiological conditions or at a lower expression level. The identification of Rab4 interacting proteins and further study on the molecular mechanism of Rab4–CFTR interaction will facilitate future studies for the elucidation of Rab4GTPase involvement in the regulation of CFTR activity under normal or diseased [60–62] physiologically important conditions.

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