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Rab27a negatively regulates CFTR chloride channel function in colonic epithelia: Involvement of the effector proteins in the regulatory mechanism

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Abstract

Cystic fibrosis, an autosomal recessive disorder, is caused by the disruption of biosynthesis or function of CFTR. CFTR regulatory mechanisms include channel transport to plasma membrane and protein–protein interactions. Rab proteins are small GTPases involved in vesicle transport, docking, and fusion. The colorectal epithelial HT-29 cells natively express CFTR and respond to cAMP with an increase in CFTR-mediated currents. DPC-inhibited currents could be completely eliminated with CFTR-specific SiRNA. Over-expression of Rab27a inhibited, while isoform specific SiRNA and Rab27a antibody stimulated CFTR-mediated currents in HT-29 cells. CFTR activity is inhibited both by Rab27a (Q78L) (constitutive active GTP-bound form of Rab27a) and Rab27a (T23N) (constitutive negative form that mimics the GDP-bound form). Rab27a mediated effects could be reversed by Rab27a-binding proteins, the synaptotagmin-like protein (SLP-5) and Munc13-4 accessory protein (a putative priming factor for exocytosis). The SLP reversal of Rab27a effect was restricted to C2A/C2B domains while the SHD motif imparted little more inhibition. The CFTR-mediated currents remain unaffected by Rab3 though SLP-5 appears to weakly bind it. The immunoprecipitation experiments suggest protein–protein interactions between Rab27a and CFTR. Rab27a appears to impair CFTR appearance at the cell surface by trapping CFTR in the intracellular compartments. Munc13-4 and SLP-5, on the other hand, limit Rab27a availability to CFTR, thus minimizing its effect on channel function. These observations decisively prove that Rab27a is involved in CFTR channel regulation through protein–protein interactions involving Munc13-4 and SLP-5 effector proteins, and thus could be a potential target for cystic fibrosis therapy.

Keywords: CFTR; Rab27a; Trafficking; Regulation; SLP-5; Munc13-4

Cystic fibrosis (CF), an autosomal recessive disorder, is caused by the dysfunction of cystic fibrosis transmembrane conductance regulator (CFTR) [1,2]. Although several CF mutations have been described, the predominant mutation is at position 508 in which the loss of phenylalanine results in a mislocalized channel in the cytoplasm [3,4]. Alternatively, excessive CFTR activity results in toxin-induced secretory diarrhea [5,6]. CFTR is involved in the regulated electrolyte transport across the luminal surfaces of a variety of epithelial cells, including the colon [7–9].

Rab GTPases have been defined in multiple tissues, are ubiquitously distributed throughout the eukaryotic system, and are characteristically implicated in regulated endocytosis, exocytosis, and secretions [10,11]. Rab proteins switch between the GTP- and GDP-bound conformations to reside either in active or inactive status [12,13]. Each Rab protein plays a highly defined, crucial, and specific responsibility in the transport process [14–16]. Moreover, Rab proteins and their effectors act upstream of the SNARE complex and regulate the initial stages of membrane tethering and fusion [17,18]. Rab27a is reportedly expressed in a broad range of specialized secretory cells, including exocrine, endocrine, ovarian, and hematopoietic cells, most of which undergo regulated

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exocytosis [19,20]. Additionally, Rab27a is the only Rab protein whose dysfunction is associated with human immunodeficiency caused by Griscelli syndrome [21] and partial albinism which reflect the dysfunction of lysosome-related organelles [20]. Rab27a is a potential pharmacological target for the glucose competency in patients with diabetes mellitus [22].

Initially, we reported Rab4 regulation of CFTR in HT-29 cells [23], which endogenously express CFTR and respond to cAMP stimulation [24,25]. In the present communication, we investigated if Rab27a, a highly potential candidate in secretions and lysosomal degradation, regulates CFTR activity. The cells were over-expressed with individual Rab isoforms, and cAMP induced but DPC inhibited currents were recorded. These currents represent CFTR-mediated currents as suggested previously [26,27]. Here we present several lines of evidence to suggest that Rab27a is a negative modulator of CFTR Cl^- channel function in HT-29 cells and the molecular mechanism that defines this regulation involves protein–protein interaction, Munc13-4, and synaptotagmin-like proteins (SLP-5) dependent pathway.

Materials and methods

Materials and reagents. Colonic epithelial HT-29 cells (HTB-38) were purchased from American type culture collection (ATCC), (Manassas, VA). The CFTR-monoclonal antibodies used in the studies were procured from Upstate USA, Inc. (Charlottesville, VA). Horseradish peroxidase-conjugated (HRP-conjugated) secondary antibodies (anti-rabbit and anti-mouse) were from Pierce Chemical Co. (Rockford, IL, USA). Chariot® protein delivery system was available from Active Motif, (Carlsbad, CA). Control and Rab27a specific SiRNA were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA).

Cell line. 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 Falcon 12- or 24-well inserts for all the experiments and maintained to determine the cAMP-dependent CFTR-mediated currents [54,55]. Medium was renewed every alternate day and the cells were subcultured with trypsin–EDTA every 5–6 days. For recording currents, the cells were grown on Falcon 24-well inserts. The cells were transfected with Rab constructs using Lipofectamine (Gibco-BRL-Invitrogen) according to the manufacturer's instructions. The expression of each construct was confirmed by Western blot analysis for each set of reactions outlined in this communication.

Measurements of short circuit current (I_{sc}). CFTR-mediated currents were recorded with EVOM™ epithelial voltohmeter using STX2 electrode (WPI, Sarasota, FL). cAMP-dependent activation was achieved by adding a cocktail containing IBMX (100 μM), dibutyryl-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 confluent monolayer was mounted in a modified Ussing chamber (Trans-24 miniperfusion chamber, Warner Instruments, Hamden, CT). Apical and basolateral chambers were continuously bathed with medium and I_{sc} were measured with transepithelial voltage clamped at 0 mV.

Cell surface protein biotinylation and CFTR detection. The experiments were performed with Sulfo-NHS-SS-Biotin utilizing the cell surface biotinylation kit (Pierce Biotechnology Inc., Rockford, IL) as per the manufacturer's directions. In short, the intact HT-29 cells were biotinylated (0.5 mg/mL) at 4 °C for 30 min. Following quenching, the cell lysates were centrifuged, and the biotinylated proteins were adsorbed on the immobilized streptavidin resin beads. The beads were isolated by gentle centrifugation, washed, and the bound proteins were eluted from the resin by the addition of SDS–PAGE sample buffer containing 5% β-mercaptoethanol and incubation at 37 °C for 1 hr. The samples were analyzed for CFTR expression by Western blot analysis using a CFTR-monoclonal antibody from Upstate USA, Inc. (Charlottesville, VA). The blots were raised using Enhanced Chemiluminescence (ECL) and the films were developed using autoradiography.

Immunoprecipitation experiments. The experiments were performed essentially as described before [23,28]. In short, the cell lysates in RIPA buffer (1% NP-40 or Triton X-100, 0.2% sodium deoxycholate, 1 mM EDTA, 0.2% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 1% Trasylol, a 1:100 dilution with 100 mg/ml leupeptin, and 100 mM phenylmethylsulfonyl fluoride) were centrifuged to recover the supernatant at 14,000-rpm for 5 min. The lysates were precleared with protein-A–Sepharose beads and the supernatants were further incubated with a specific antibody overnight at 4 °C followed by incubation with Sepharose beads (Sigma) for 2 more hours. The beads were solubilized in SDS sample buffer and analyzed by SDS–PAGE. The proteins were electroblotted to a PVDF membrane and incubated with antibody for 60 min at RT. The bound antibody was detected by ECL and quantitated by phosphor-imaging under conditions where there was a linear relationship between intensity and pixel number. In some cases, the blots were reused by stripping the bound antibodies and re-probed with additional antibodies. For this, membranes were immersed in stripping buffer (62.5 mM Tris–HCl (pH 6.7), 2% SDS, and 100 mM β-mercaptoethanol) for 30 min at 55 °C, and washed extensively in Tris-buffered saline (0.1 M, pH 7.4, 0.05% Tween 20) at RT. Membranes were re-blocked in milk-TBS, incubated with the desired antibody, and raised for protein detection using ECL as described above.

SiRNA studies. The control and SiRNAs specific for Rab isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The control siRNA used in the study had a non-targeting 20–25 nucleotide siRNA designed as a negative control. The SiRNA effect was confirmed by Western blot analysis of transfected cells. 100 nM SiRNA (final conc.) was mixed with 20 μL of the transfection medium reagent per well and incubated at RT. In another vial, 1.2 μL of the transfection reagent was mixed with 20 μL transfection medium and incubated. The contents of both vials were mixed. After 20 min, the contents were mixed with 160 μL of the transfection medium and this solution was laid on top of the cells in each well. The cells growing on inserts in a 24-well plate (~50% confluent for this experiment) were used for this experiment. The plate was incubated in 37 °C with 5% CO_2 for 5–7 h, and then 200 μL of the culture medium with double serum was added on top of the inserts. The CFTR-mediated currents were recorded 24 h post-SiRNA transfection as described before.

Purification of SLP. PGEX-SLP-C2A, C2B, and SHD proteins were isolated from bacterial cultures expressing the respective cDNA. The proteins were purified using glutathione beads (Amersham-Glutathione Sepharose™ 4B) using the conventional method. The beads in phosphate-buffered saline (0.15 M, pH 7.3), with 50% slurry of beads, were incubated with bacterial lysates at 4 °C overnight. The beads were washed with PBS and RIPA buffer. The SLP proteins were eluted by adding 100 μl elution buffer (50 mM Tris, 10 mM glutathione, pH 8.0) to the beads, incubated for 10–15 min at RT, and then centrifuged to collect the supernatant. The protein concentration was determined by bicinchoninic acid (BCA) assay.

Introduction of proteins using Chariot® delivery system. The experimental procedure has previously been defined [23,28]. In short, SLP were mixed in a complex with the Chariot® reagent (diluted in 70% DMSO) at a ratio of 20 μg protein: 10 μl in 100 μl PBS for 30 min at RT. The protein:Chariot® complexes were overlaid onto cultured cells in the presence of serum free medium and incubated at 37 °C for 1 h followed by incubation for 2 more hours in presence of complete growth medium.

Isolation of plasma membrane and intracellular CFTR. The intracellular and cell surface CFTR populations were obtained essentially by the method described previously for MDCK cells [29]. Cells grown on filters or plates were induced, surface biotinylated, quenched, and finally scraped in PBS. Cells were pelleted and lysed for 30 min on ice. The samples were centrifuged and the supernatants were collected. For CFTR at the cell

surface, biotinylated proteins were adsorbed on 50% streptavidin–agarose bead slurry by rotating for 2 h at 4 °C. After brief centrifugation, supernatants representing the intracellular pool were collected and processed accordingly. Proteins were quantified by BCA method, separated by SDS–PAGE, and transferred to nitrocellulose for immunoblotting. The blots were raised for CFTR detection 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

Effect of Rabs on CFTR Cl^- currents

To test the hypothesis that Rab proteins functionally modulate CFTR chloride channel activity, CFTR-mediated cAMP-induced and DPC-inhibited currents were assayed in HT-29 cells by over-expressing different Rab isoforms (Fig. 1, middle). These cells natively express functional CFTR channel and produce cAMP-inducible currents that can be inhibited by the addition of 1 mM DPC to the bath solution. These represent CFTR-mediated currents since the introduction of CFTR specific SiRNA but not the control SiRNA completely eliminated these recordings (Fig. 1, top). The CFTR Cl^- currents were inhibited by Rab27a, however, two other isoforms, Rab3 and Rab5, failed to impose any modulation. Rab27a inhibited basal and CFTR currents. An average of $66 \pm 14\%$ inhibition of CFTR currents was observed when 0.5 μg is expressed. However, the over-expressed Rab27a at a concentration >2 μg completely blocked CFTR-mediated currents (Fig. 1, bottom). Rab5 inhibited basal currents by about 45%, but did not seem to affect CFTR-mediated currents. Over-expression of Rab3, however, could not modify basal or cAMP-induced currents. Fig. 1 represents only the CFTR-mediated currents (cAMP-induced and DPC inhibited) while the basal (non-cAMP-induced but DPC inhibited) currents were not shown considering the specificity of CFTR activity. These data indicate that functional regulation of CFTR by Rab27a is an isoform-specific phenomenon. The data further suggest that Rab27a inhibition of CFTR-mediated currents does not depend on GTP/GDP status as both mutants imparted inhibition though the GTP-locked form (Q78L) appeared more aggressive (Fig. 1, middle).

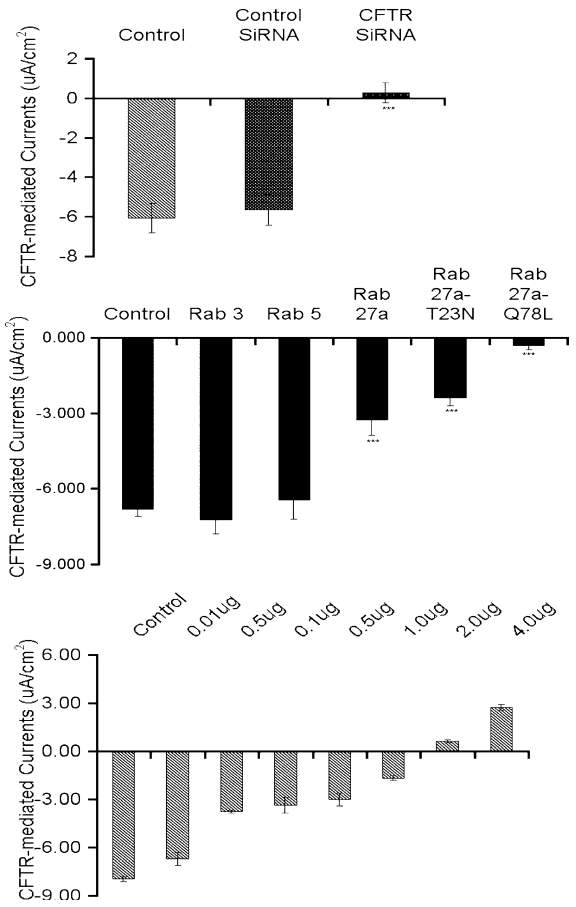

Fig. 1. Rab isoforms regulate CFTR-mediated currents in HT-29 cells. HT-29 cells grown on cell inserts were transfected with wild-type Rab constructs using lipofectamine reagent. The CFTR-mediated currents were recorded two days later by incubating the cells in cAMP cocktail (IBMX, 100 μM; dibutyryl cAMP, 200 μM; and forskolin, 20 μM) for 15 min followed by inhibition with DPC (1 mM) as described in the text. The data represents means of three individual experiments. Currents recorded with (top) control and CFTR specific SiRNA, (middle) different Rabs; (bottom) with increasing concentration of Rab27a. Experimental conditions that resulted in a significant change ($p > 0.05$) from the relevant control values are denoted by the asterisk.

Effect of SiRNA delivery on CFTR Cl^- currents

Transfection of HT-29 cells with Rab27a SiRNA led to over 100% activation of CFTR-mediated currents, as compared to the control (Fig. 2). Control SiRNA contained a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. This measure led us to define the specific effect of Rab27a on CFTR-mediated currents. We also defined the level of Rab expression by Western blot analysis in the presence of isoform specific SiRNA (Fig. 2B and C). Introduction of SiRNA in our conditions enabled us to inhibit Rab expression between 70% and 80%. Although, we could not completely abolish the Rab expression, the level of inhibition by SiRNA is large enough to define their effect on CFTR functional activity. Rab5a SiRNA could not alter CFTR-mediated currents (data not shown). These observations indicate that Rab27a functionally regulates CFTR activity in HT-29 cells.

Immunoprecipitation of CFTR with Rab proteins

The functional data suggested the possibility of physical interactions between CFTR and Rab proteins. In order to examine this hypothesis, we performed co-immunoprecipitation studies in which HT-29 cells expressing Rab isoform proteins (confirmed first by Western blot analysis) were immunoprecipitated with isoform-specific anti-Rab antibody and probed with CFTR antibody. Alternatively, we utilized anti-CFTR antibody to immunoprecipitate Rab

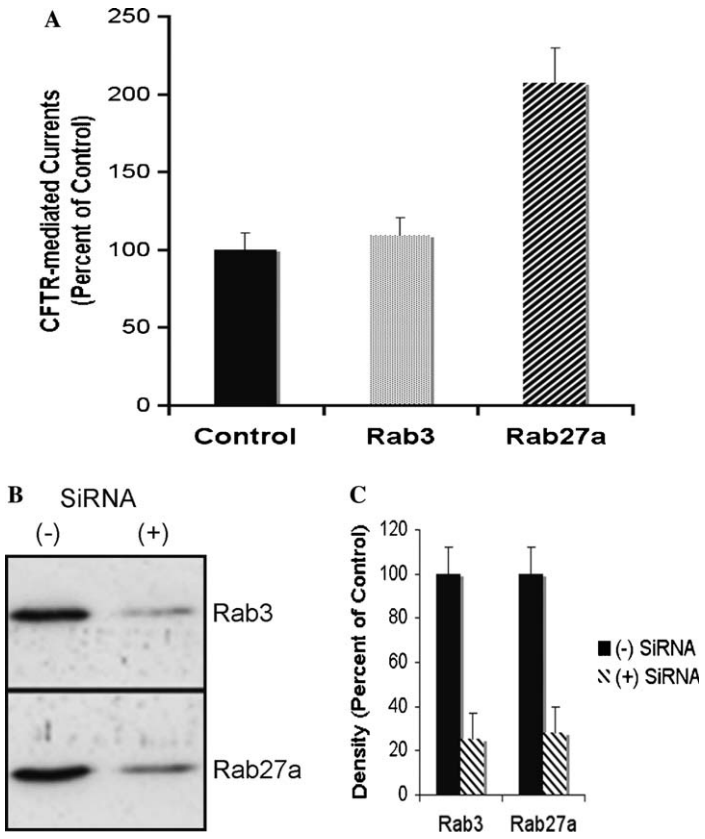

Fig. 2. Introduction of specific SiRNA reverses inhibition of CFTR-mediated currents. The HT-29 cells were targeted with SiRNA (final conc. 100 ng) specific for Rab27a as described in the text. CFTR-mediated currents were measured 24 h post-SiRNA transfection. (A) CFTR-mediated currents recorded in presence of isoform specific SiRNA, (B) Western blot analysis of Rabs from protein lysates showing SiRNA inhibits Rab expression, and (C) Densitometric analysis of Rab proteins from (B). For each Rab isoform (−) SiRNA is treated as 100%. The result is the average of three individual experiments.

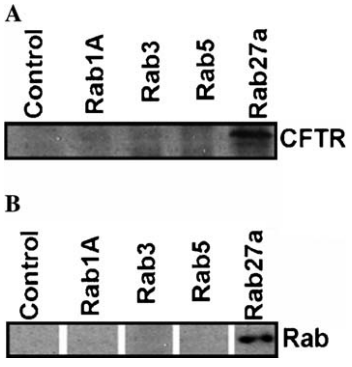

Fig. 3. Rab27a interacts with CFTR in HT-29 cells. (A) Isoform specific anti-Rab antibodies were used to immunoprecipitate CFTR proteins from HT-29 cell lysates expressing Rab isoforms. The immune complex was adsorbed on protein-A–agarose beads and then separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membrane. The blots were probed with anti-CFTR antibody. (B) The protein lysates were immunoprecipitated with anti-CFTR antibody and probed with isoform specific Rab antibodies. IgG was used as control in all experiments. The data show interaction of Rab27a with CFTR in vivo and point to its involvement and physiological significance in the regulation of CFTR in native cells.

proteins and decorated the immunoblots with Rab antibodies. Our observations indicated physical interactions of CFTR with Rab27a (Fig. 3A) but other Rab isoforms failed to produce convincing results. Similar data were observed when the native CFTR was first precipitated with anti-CFTR antibody followed by immunodetection with Rab isoform-specific antibodies (Fig. 3B). These data suggest isoform-specific CFTR-Rab interaction in the HT-29 colonic epithelial cells.

Rab27a decreases CFTR plasma membrane expression

Considering the involvement of Rab proteins in cell compartmental trafficking, we next explored the hypothesis that the reduction in CFTR-mediated currents in the cells over-expressed with Rab27a protein was due to reduced CFTR at cell surface. We biotinylated the cell surface proteins by employing cell impermeant Sulfo-NHS-SS-biotin and pulled down the complex with streptavidin–agarose. In line with our hypothesis, we observed reduction in the abundance of CFTR in the cells expressing Rab27a (Fig. 4). These observations were restricted to only these isoforms as expression of Rab3 or Rab5 could not effect changes in CFTR density. These data demonstrate that Rab27a modulates CFTR transport to the cell surface by either retaining the channel proteins intracellularly or incapacitating its exocytosis to the cell membrane.

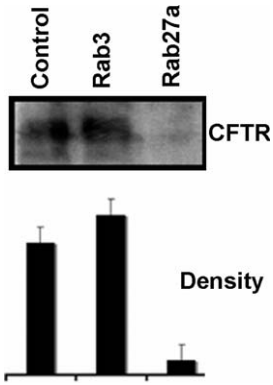

Fig. 4. Rab27a alters CFTR cell surface expression. HT-29 cells (60–70% confluent) grown on cell inserts were transfected with wild-type Rab isoform constructs. Cell surface proteins were biotinylated with cell impermeant Sulfo-NHS-SS-biotin, pull down with streptavidin–agarose, separated by SDS–PAGE and transferred to PVDF membrane by Western blotting. The blots were probed with CFTR antibody (upper panel). The data reflect lower expression of CFTR in HT-29 cells expressing Rab27a as depicted by densitometric analysis in the lower panel (data are means of three individual experiments).

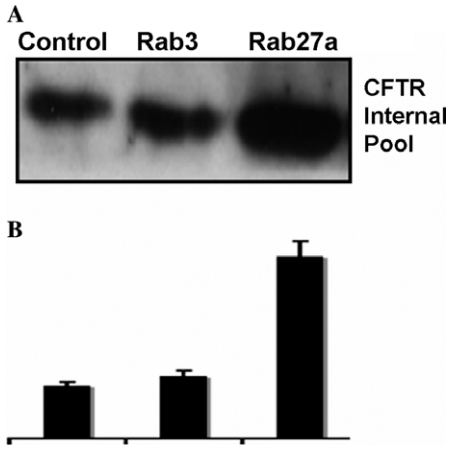

Fig. 5. Rab27a increases CFTR internal (cytoplasmic) pool. HT-29 cells were transfected with wild-type Rab isoforms. The cytosolic proteins representing non-biotinylated proteins were isolated as described in the text and separated by SDS–PAGE and transferred to PVDF membrane. The blots were probed with CFTR antibody (A). The data reflect higher expression of CFTR in HT-29 cells transfected with Rab27a as depicted by densitometric analysis in (B) (data are means of three individual experiments).

Rab27a increases internal or cytosolic CFTR pool

Since we observed a considerable reduction in the membrane localized CFTR with Rab27a transfection, we reasoned that it might be due to the increased abundance of CFTR in the cytosol. This is possible if these Rab isoforms impair CFTR transport to the cell membrane. In order to achieve this objective, we isolated soluble plasma (cytosolic) proteins from HT-29 cells transfected with Rabs and analyzed the presence of CFTR by Western blot analysis (Fig. 5). Our data indicate that Rab27a increases the cytosolic or internal pool of CFTR. A similar observation was not made with Rab3, suggesting that Rab27a imparts a specific effect on CFTR trafficking to the plasma membrane.

Munc13-4 reverses Rab27a inhibition of CFTR-mediated currents

Several recent studies including ours [20,30] suggest that Munc13-4 is a highly specific binding partner of Rab27a. Accordingly, we hypothesized that Rab27a might modulate CFTR function by utilizing this effector protein. In order to specify this, we co-transfected HT-29 cells expressing Rab27a with Munc13-4 and recorded the CFTR-mediated currents (Fig. 6). This maneuver resulted in the reversal of Rab27a effect. In order to define the mechanism, we performed the immunoprecipitation studies. Our data suggest that Rab27a weakly binds to CFTR in the presence of Munc13-4, possibly due to the higher affinity of Rab27a for Munc13-4 than for CFTR. These data indicate that Rab27a inhibits CFTR activity by protein–protein interactions and the diminished interaction with CFTR in the presence of Munc13-4 reverts its negative effect on channel function.

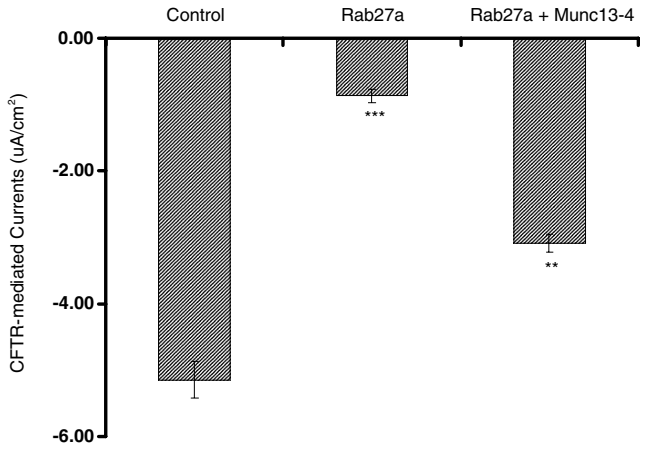

Fig. 6. Munc13-4 reverses Rab27a inhibition of CFTR-mediated currents. HT-29 cells were transfected with wild-type Rab27a, and Munc13-4 and CFTR-mediated currents were recorded 48 h post-transfection as a cAMP-induced but DPC-inhibited component as described earlier. The data is a mean of individual experiments performed. A significant change of Rab27a ($p > 0.05$) from the control values is denoted by three asterisk while Munc13-4 (two asterisk) showed a significant change from Rab27a (middle bar).

Synaptotagmin like proteins (SLP-5) partially reverse CFTR inhibition by Rab27a

SLP-5 proteins have been characterized as specific binding partners of Rab27a. These proteins are characterized by the presence of C2A and C2B as membrane binding motifs, where as SHD domain is essentially involved in Rab27a interaction. By the introduction of these proteins by Chariot® delivery system, the reversal of CFTR-mediated currents was noticed with constructs carrying only C2A and C2B domains, while SHD motif expression

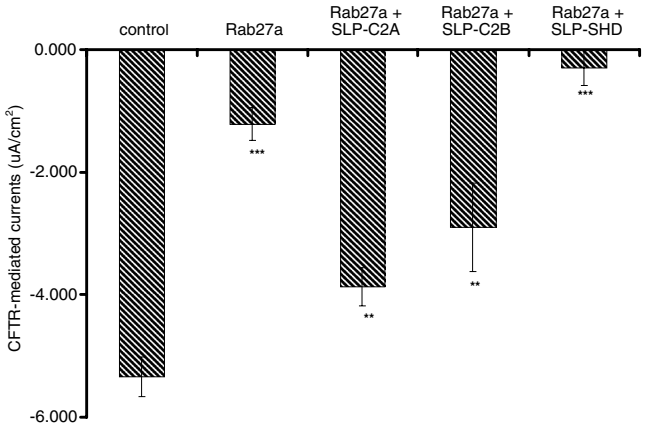

Fig. 7. Rab27 dependent inhibition is reversed by synaptotagmin-like protein (SLP-5). The purified Slp proteins were introduced into HT-29 monolayers using Chariot® delivery system. SLP-GST proteins, purified using glutathione and followed by dialysis, were mixed in a complex with Chariot reagent at a ratio of 20 μg protein: 10 μL in 100 μL of PBS (pH 7.3) and the protein:Chariot® complexes were overlaid onto cultured cells in serum free medium and incubated at 37 °C for 1 h followed by two more hours in presence of complete medium. The CFTR-mediated currents were recorded 48 h later. Data are a mean of six individual wells under each condition. A significant change ($p > 0.05$) from the control values is denoted by three asterisk while values with two asterisk denote a significant change from Rab27a (second bar) inhibited currents.

exhibited little more inhibition (Fig. 7). The reversal of currents by C2A or C2B motifs suggests the necessity of the membrane component for the attachment of the CFTR complex in addition to the requirement of SHD for Rab27a binding. The higher inhibition of CFTR-mediated currents recorded with SHD domains further illustrates the significance of membrane attachment. These Rab27-effector proteins provide a crucial understanding of the molecular mechanism by which Rab27 regulates CFTR trafficking to the plasma membrane.

SLP-5 and Munc13-4 impair CFTR ability to interact with Rab27a

In order to assess the mechanism by which Rab27a-binding proteins, SLP-5 and Munc13-4 regulate CFTR function, we transfected cells with Rab27a and one of these proteins followed by immunoprecipitation with Rab27a antibody, and then probed the immunoblots with CFTR antibody. Our data suggest that the presence of SLP-5 and Munc13-4 significantly diminishes the CFTR density compared to the control, suggesting that these effector proteins eliminate CFTR and Rab27a interaction (Fig. 8). Concomitantly, we observed intense interaction of Rab27a with SLP-5 (data not shown).

SLP and Munc13-4 redeem CFTR expression at the cell surface

Our previous observations led us to hypothesize that the over-expression and the higher binding affinity of SLP-5 and Munc13-4 with Rab27a might promote the expression

Fig. 8. SLP-5 and Munc13-4 dissociate Rab27a from CFTR. SLP-5 and Munc13-4 proteins were introduced in HT-29 cells expressing Rab27a by Chariot® delivery system while Rab27a–SiRNA (Si) was introduced in the cells by transfection. Cell lysates in RIPA buffer were centrifuged to recover the supernatant and the lysates were incubated with protein-A–Sepharose beads and Rab27a antibody for 24 h at 4 °C. The beads were solubilized in SDS sample buffer and run on SDS–PAGE, transferred to a PVDF membrane. The blots were probed with CFTR antibody and raised with ECL and the films were developed using autoradiography.

Fig. 9. SLP and Munc co-expression recycles CFTR back on plasma membrane. SLP-5 and Munc13-4 proteins were expressed in HT-29 cells transfected with Rab27a by the Chariot® delivery system and transfection respectively. Two days later, the cells were washed with PBS, and then incubated with cell impermeant Sulfo-NHS-SS-Biotin (0.5 mg/mL) at 4 °C for 30 min. After 30 min of incubation, the reaction was stopped by quenching, and the cells were solubilized in RIPA buffer. The lysates were centrifuged to collect the supernatant. Equal amounts of the lysate from each sample were mixed with 200 μL of streptavidin–agarose beads and incubated overnight at 4 °C. The beads carrying biotinylated proteins were washed with RIPA buffer, eluted by the addition of SDS-sample buffer, and analyzed by SDS–PAGE. The samples were analyzed by Western blot analysis using CFTR-monoclonal antibody. The blots were raised using enhanced chemiluminescence (ECL) and the films were developed using autoradiography.

of CFTR at cell surface due to the limited availability of Rab27a to interact with CFTR. Biotinylation with the cell impermeant reagent suggested a reduction in the CFTR abundance on the membrane in cells expressing Rab27a (Fig. 9). However, the over-expression of SLP-5 or Munc13-4 redeems the apical CFTR expression. These data further support our hypothesis that Rab27a modulates CFTR transport to the cell surface by retaining the channel protein intracellularly. SLP-5 and Munc13-4, on the other hand, due to their stronger binding affinity for Rab27a, hinder the CFTR-Rab27a interaction and therefore channel density at the plasma membrane is restored, resulting in the reversal of CFTR-mediated currents.

Discussion

Cystic fibrosis transmembrane conductance regulator (CFTR)-dependent chloride (Cl^-) secretion in human colonic epithelia cells like HT-29 and T84 has been well defined and extensively reported [31–33]. Our study utilized HT-29 cells to determine the role of Rabs in the regulation of CFTR-mediated Cl^- currents. CFTR-mediated DPC-inhibited currents reported in this study could be completely

eliminated in the presence of CFTR-specific SiRNA. The principle observations of this study suggest: (i) CFTR function is negatively regulated by Rab27a, (ii) CFTR physically interacts with Rab27a, (iii) which impairs CFTR expression at the plasma membrane resulting in reduced CFTR at the apical membrane while increasing cytosolic CFTR pool, (iv) Rab27a dependent inhibition of CFTR could be reversed by its binding partners, Munc13-4, and synaptotagmin-like proteins (SLP-5), who (v) impair Rab27a ability to interact with CFTR and (vi) re-establish CFTR expression at the plasma membrane.

We recently reported formidable CFTR chloride and amiloride-sensitive epithelial sodium channel (ENaC) currents in HT-29 cells [23,28,34]. In these cells, we could completely wipe out CFTR-mediated currents by introducing CFTR–SiRNA indicating that the currents recorded by us are 100% CFTR-dependent (Fig. 1).

The trafficking and the assembly of the cystic fibrosis transmembrane regulator (CFTR)-chloride channel is critical in understanding how ion channels and ABC transporters are formed and also in dissecting the misassembly of CFTR in cystic fibrosis [3,35,36]. Under normalized physiologic conditions, native CFTR recycles from early endosomes back to the cell surface, whereas misfolding prevents recycling and facilitates lysosomal targeting [37]. Folding, maturation, and trafficking of transmembrane proteins from the ER to their ultimate destination plasma membrane is a regulated translocation of transport vesicles [38,39]. Subsequently, vesicular fusion with target membrane results in increased channel density at the cell surface. The molecular mechanisms that regulate the translocation of these vesicles reside in the ability of multiple Rabs to control the regularized traffic [10,40].

By using colonic epithelial cells HT-29, we have demonstrated that Rab27a acts as a negative regulator of CFTR function. This effect is specific since Rab3 and Rab5 show no change. The isoform specific modulation of CFTR was confirmed (a) by over-expressing Rab proteins, (b) and by silencing the de novo expression of Rabs by SiRNA. We also observed that the introduction of Rab27a antibody by Chariot® delivery system abolished the inhibitory effect of Rab27a on CFTR-mediated currents (data not shown). We further established that Rab27a physically interacts with CFTR as demonstrated by the immunoprecipitation experiments (Fig. 3). In the studies published earlier it was shown that surface CFTR enters several different routes; one of which includes a Rab5-dependent initial step to early endosomes [41,42] or Rab11a-specific apical recycling compartment. Our study does not address the intracellular or compartmental trafficking, and our experimental conditions are entirely different from the one used in above studies.

The biotinylation studies [43,44] with cell impermeant biotin clearly demonstrate that CFTR density is compromised in the cell membranes of HT-29 cells actively expressing Rab27a, suggesting the involvement of these proteins in CFTR transport or trafficking. The increased abundance of the internal CFTR pools provides evidence that Rab27a [20] is involved in the regulated membrane trafficking of CFTR. Moreover, studies with S9 cell line derived from the bronchial epithelia of a cystic fibrosis patient and corrected for CFTR expression by stable transfection also confirm the inhibition and protein–protein interaction of CFTR by Rab27a. The expression of Δ508 in Chinese hamster ovary (CHO) cells also resulted in its interaction with Rab27a (data not shown), suggesting Rab27a potential in cystic fibrosis therapy.

Rab27a appears to act by recruiting the effector proteins and several such binding partners have been described. For example, Munc13-4 has been purified from the platelet cytosolic fraction [45]. Munc13-4 is hypothesized to contain a novel Rab27-binding domain, although it lacks an N-membrane-binding domain. Munc13-4 is a direct partner of only Rab27a and it does not bind to any other GTPases [45]. Both Rab27a and Munc13-4 have been shown to preferentially localize on secretory lysosomes in several tissues and cell line cells [46]. Our data, with both CFTR and ENaC [30], led us to believe that the Rab27a/Munc13-4 complex is an essential regulator of channel function in the plasma membrane of epithelial cell system, and might be similar to what has been observed in mast cells, platelets, and histamine release [47].

The synaptotagmin-like protein (SLP-5), though exceptionally, interacts with several Rab isoforms including Rab3 and Rab27a in vitro, but it preferentially interacts with Rab27a in living cells [48,49]. Like other members, SLP-5 has an N-terminal SLP homology domain (SHD) and C-terminal tandem C2A and C2B domains separated by a linker sequence [50]. SHD motifs are involved in protein–protein interaction with Rab27a [51], while C2A or C2B are putative membrane binding domains. Rab27 and its effector form a core with syntaxin 1a and Munc18-1, which essentially categorizes it as major plasma membrane trafficking protein. SLP-5 appears to interact with both Rab27a (T23N), a dominant negative GDP-locked form, and Rab27a (Q78L), a dominant active or GTP-locked form [52].

In conclusion, the negative regulation of CFTR function by Rab27a is the consequence of depleted appearance of the channel at the plasma membrane. Given the Rab27a association with exocytosis and lysosomal trafficking [52–54], it is likely that the major CFTR pool is destined to the lysosome in the cells over-expressing Rab27a. Our studies suggest that CFTR and Rab27a predominantly colocalize in three compartments in the following order; plasma membrane < microsomes < lysosomes (data not shown) suggesting that Rab27a impairs CFTR expression at the plasma membrane. Munc13-4 (a priming exocytosis factor) and SLP-5 both reverse the inhibitory effect of Rab27a by preferentially associating with Rab27a and depriving its ability to bind CFTR. Our immunoprecipitation studies confirm this possibility. The results with the C2A or C2B domains of SLP-5 indicate that in addition to the protein–protein interaction, the effect of Rab27a necessitates

the presence of a membrane binding domain, since in the absence of either one of the C2 domains the CFTR-mediated currents were further down-regulated.

The Rab27a/effector system is fast emerging as key regulator of several essential functions since it is reportedly involved in the glucose-specific signals for the exocytosis of insulin granules in pancreatic β cells [55]. Though Rab27a is essentially a lysosome-related trafficking protein, its association with the regulation of various exocytotic pathways is fast emerging [52]. In view of the significant role of Rab27a in the pathogenesis of Griscelli syndrome [56], where the Rab27a/Munc13-4 complex acts as an essential regulator of secretory granule fusion with the plasma membrane in cytotoxic T lymphocytes [46], we presume that Rab27a exhibits essentially similar credentials in epithelial cell systems controlling the exocytosis of ion channels. Moreover, we anticipate a more complex phenomenon of Rab27a-associated CFTR regulation with a well orchestrated involvement of Rab27a-binding proteins like SLP4, Slac-2, Noc2 or rabphilin, etc., in the bronchial or intestinal epithelia where CFTR is natively regulated. Although our studies did not address a vital issue related to CFTR mutants like Δ508, which display impaired trafficking and assembly, these studies are vital to explore the possibility of Rab27a involvement in the regulated exocytosis of diseased forms. It is likely that Rab27a with its effector proteins may provide a therapeutic approach to regulating CFTR activity associated with cystic fibrosis.

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