Rab11b regulates the trafficking and recycling of the epithelial sodium channel (ENaC)

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The epithelial sodium channel (ENaC) constitutes the rate-limiting entry step in Na+ reabsorption across several epithelia including the kidney, lung, colon, salivary glands and sweat glands (8, 27, 45). ENaC-mediated transport is an essential component underling salt and water homeostasis. Knockout of the ENaC α-subunit in mice is lethal as offspring fail to clear airway fluid at birth (32). Abnormal regulation of the channel that results in either gain or loss of function has been implicated in the pathogenesis of several disease states, including forms of salt-sensitive hypertension, salt wasting (pseudohypoaldosteronism type II), and is thought to contribute to the progression of pulmonary disease in cystic fibrosis (28, 33, 43, 51, 63).

The apical membrane abundance and open probability of ENaC are altered by a wide variety of hormonal and cellular effectors (6, 7, 30, 42, 48, 52). The hormone vasopressin acutely increases Na+ transport by increasing the apical surface density (channel number) of ENaC (9, 12, 15, 21, 22, 39, 66). These channels are transported from subapical vesicles and inserted into the apical membrane by exocytosis (9, 11). To regulate the surface ENaC density, ubiquitination of ENaC at the apical surface acts as a signal for channel endocytosis (1, 19, 24, 28, 34, 37). We previously demonstrated the involvement of endocytic adaptor epsin in the clathrin-mediated retrieval of ubiquitinated ENaC from the apical membrane (68). These endocytosed channels traffic through EEA1-positive early endosomes (68). Ubiquitinated cargo such as ENaC is recruited to early endosomes by the ubiquitin-interacting motifs on hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and other components of the endosomal-sorting complexes required for transport (ESCRT-0) complex (17). Hrs has recently been shown to be critical to ENaC recycling (72). Retrieved channels in EEA1 compartments are only free to recycle back to the apical cell surface once the ubiquitin has been removed by specific deubiquitinating proteins (DUBs). Two DUBs have been implicated in this process to date (10, 23), but it is likely that more DUBs will be implicated in this regulation. Our previous work demonstrated that the DUB UCH-L3 appears to act on ENaC in the early endocytic pathway to facilitate its recycling. The itinerary of ENaC trafficking through intracellular vesicle compartments following the fate decision in early endosomes has yet to be mapped, but from a growing body of work it is likely that the small GTPases will be important mediators of ENaC’s intracellular trafficking.

The Rab-GTPase family of proteins is a subset of the larger Ras superfamily of G proteins and comprises >70 family members (26, 61). In the process of transitioning between donor and acceptor membrane compartments, Rab proteins switch between two conformations, an inactive, GDP-bound and an active, GTP-bound form (47). An exchange factor (GEF) catalyzes the GDP-GTP transition while GTP hydrolysis to GDP is catalyzed by a GTPase-activating protein (GAP) (26, 29). Rab proteins facilitate steps in vesicle regulation, including vesicle formation, trafficking, and fusion. They are often linked to cytoskeletal motor proteins to regulate vesicle movement (18, 29, 46). It is possible to alter the intrinsic activity of RabS by introducing mutations that lock the GTPase in the GTP-bound (constitutively active, CA) or the GDP-bound (dominant negative; DN) confirmation.

A number of epithelial channels have been shown to be regulated by Rab proteins, providing precedence for a role of RabS in channel regulation by membrane trafficking (14, 49, 53–58, 65, 71). As ENaC recycles in polarized epithelial cells, it is likely that the channel traverses a number of Rab-dependent compartments en route to the apical membrane. Prior studies have demonstrated the involvement of Rab4, 11, and 27 in ENaC regulation (38, 53, 54, 58). These Rabs have been previously shown to regulate exocytosis of recycling and biosynthetic cargoes (20, 25, 35, 36, 50, 59, 60, 70).

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The two related isoforms of the GTPase Rab11, Rab11a and Rab11b, share ~90% amino acid homology, with the least similarity found in their membrane-binding C termini (40). Rab11a localizes to the apical recycling endosome (ARE) in polarized epithelial cells, where it regulates the apical recycling and exocytic insertion of membrane proteins (13, 16). Rab11b localizes to apical vesicles distinct from a Rab11a compartment in polarized MDCK and gastric parietal cells (41). While a recent publication implicated Rab11a in the compartment in polarized MDCK and gastric parietal cells (13, 16). Rab11a localizes to the apical recycling endosome (ARE) in polarized epithelial cells, where it regulates the apical recycling and exocytic insertion of membrane proteins (13, 16). Rab11a, share similarity found in their membrane-binding C termini (40).

Disruption of Rab11 activity with DN mutants led to a reduction in ENaC-mediated Na transport. Specific knockdown of Rab11a using small interfering (si) RNAs produced a small decrease in ENaC surface expression and no significant decrease in ENaC-mediated Na transport. A much larger impact on ENaC regulation was obtained when Rab11b activity was altered. With both the expression of a DN-Rab11b mutant or knockdown of endogenous Rab11b, basal, unstimulated Na transport was reduced and acute ENaC trafficking and recycling to the apical surface were significantly impaired. These findings indicate the absolute requirement for Rab11b to deliver ENaC to the apical membrane.

MATERIALS AND METHODS

Reagents and antibodies. All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The anti-Rab11b and α1-Na-K-ATPase polyclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA). Other antibodies used included anti-Rab11a (BD Biosciences, San Jose, CA), anti-GFP (Abcam, Cambridge, MA), anti-actin (Sigma), monoclonal anti-β-ENaC (Santa Cruz Biotechnology, Santa Cruz, CA), anti-γ-ENaC antibody (Stress-Marq, Victoria, BC), and fluorescently tagged phalloidin-Alexa 633 (Invitrogen, Carlsbad, CA).

ENaC antibody characterization. An affinity-purified rabbit polyclonal antibody was raised against α-ENaC using a 14-aminoc acid antigen specific for mouse α-ENaC. The sequence (PMQGGLKGD-KREEQ) was conjugated to KHL by the addition of a terminal cystine and antibodies raised and purified commercially by GenScript (Piscataway, NJ). To verify the specificity of a newly developed anti-α-ENaC antibody, Fisher rat thyroid (FRT) epithelial cells were transiently transfected with epitope and fluorescently tagged ENaC and untagged ENaC plasmids for green fluorescent protein (GFP)-α-ENaC, untagged mouse β-, γ-ENaC only, and hemagglutinin (HA)-tagged mouse α-ENaC with untagged β-, γ-ENaC. Whole cell lysates from these samples were loaded onto gels, and proteins were resolved by SDS-PAGE. Blots were probed with i) the α-ENaC antibody (1:1,000), ii) anti-HA antibody (1:1,000), iii) the α-ENaC antibody preincubated with the immunizing peptide (1 μg/ml), and iv) anti-β-actin (1:1,000) as a loading control. From the detected bands, it is clear that the antibody recognizes the full-length α-ENaC and not β- or γ-ENaC. The addition of a GFP tag shifted the apparent weight up by ~20 kDa as expected, and use of the anti-HA antibody produced a band at the same weight, indicating that the antibody is recognizing α-ENaC specifically. In addition, preincubation with the immunizing peptide eliminated the specific signal. It is therefore likely that this antibody is specific for α-ENaC in the mpkCCD cells.

B: to demonstrate the specificity of fluorescent immunolabeling, mpkCCD cells cultured on filters were fixed (as described in MATERIALS AND METHODS) and permeabilized, and indirect immunofluorescent labeling was performed using an Alexa 568 anti-rabbit secondary antibody (Invitrogen) at 1:2,000 dilution with the following conditions: i) anti-α-ENaC as the primary antibody, ii) no primary antibody (to test for nonspecific secondary labeling), and iii) anti-β-actin antibody preincubated with immunizing peptide (as for the Western blot in A). Bar = 10 μm.

Fig. 1. Characterization of a novel anti-α-epithelial sodium channel (ENaC) antibody. A: to test the specificity of the affinity-purified antibody, Fisher rat thyroid (FRT) cells were transiently transfected with the following constructs: green fluorescent protein (GFP) alone, GFP-tagged mouse α-ENaC, untagged mouse β-, γ-ENaC only, and hemagglutinin (HA)-tagged mouse α-ENaC with untagged β-, γ-ENaC. Whole cell lysates from these samples were loaded onto gels, and proteins were resolved by SDS-PAGE. Blots were probed with i) the α-ENaC antibody (1:1,000), ii) anti-HA antibody (1:1,000), iii) the α-ENaC antibody preincubated with the immunizing peptide (1 μg/ml), and iv) anti-β-actin (1:1,000) as a loading control. From the detected bands, it is clear that the antibody recognizes the full-length α-ENaC and not β- or γ-ENaC. The addition of a GFP tag shifted the apparent weight up by ~20 kDa as expected, and use of the anti-HA antibody produced a band at the same weight, indicating that the antibody is recognizing α-ENaC specifically. In addition, preincubation with the immunizing peptide eliminated the specific signal. It is therefore likely that this antibody is specific for α-ENaC in the mpkCCD cells. B: to demonstrate the specificity of fluorescent immunolabeling, mpkCCD cells cultured on filters were fixed (as described in MATERIALS AND METHODS) and permeabilized, and indirect immunofluorescent labeling was performed using an Alexa 568 anti-rabbit secondary antibody (Invitrogen) at 1:2,000 dilution with the following conditions: i) anti-α-ENaC as the primary antibody, ii) no primary antibody (to test for nonspecific secondary labeling), and iii) anti-β-actin antibody preincubated with immunizing peptide (as for the Western blot in A). Bar = 10 μm.

able to detect cleaved forms of α-ENaC in these overexpressing FRT cells by using either the anti-ENaC antibody or the HA-tagged form of the channel.

Cell culture. The mpkCCDc14 cells (provided by A. Vandewalle and M. Bens, Institut National de la Santé et de la Recherche Médicale, Paris, France) were grown in flasks (passage 30–40) in defined medium as described previously (9, 67). Growth medium was composed of equal volumes of DMEM and Ham’s F12 supplemented with 60 nM sodium selenate, 5 mg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 g/ml insulin, 20 nM p-glucose, 2% vol/vol FCS, and 20 nM HEPES (Invitrogen, Sigma), pH 7.4, at 37°C in 5% CO2–95% air atmosphere. The medium was changed every second day. For experiments, the mpkCCD cells were subcultured onto permeable filter supports (0.4-μm pore size, 0.33- and 75-cm² surface area; Transwell, Corning, Lowell, MA). Cells were cultured for at least 7
days in defined medium after which a confluent transporting cell monolayer had developed that could be assessed by recording open-circuit voltage and transepithelial resistance using "chopsick" electrodes (Millipore, Billerica, MA). Typically, 24 h before use in any investigation, medium incubating cells on filters was replaced with a minimal medium (without drugs or hormones) that contained only DMEM and Ham’s F12.

**Immunooisolation of Rab11-positive endosomes.** The immunooisolation method used was described previously (62). Briefly, mpkCCD cells cultured on 75-mm-diameter filters, washed twice with PBS at 4°C, and then scraped in 300 μl of homogenization buffer which contained 3 mM imidazole, pH 7.4, 250 mM sucrose, 0.5 mM EDTA, and Complete EDTA-free Protease Inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Cells were homogenized by 20 strokes of a Dounce homogenizer and centrifuged for 10 min at 3,000 g. The resulting postnuclear supernatant (PNS) was adjusted to a 40% (wt/vol) sucrose solution. The PNS was placed in 12 ml capacity poly- clear centrifuge tubes (Sorvall, Newtown, CT) and overlaid with 6 ml of 35% (wt/vol) sucrose and 4 ml of 25% (wt/vol) sucrose, then centrifuged in a TH-641 rotor at 108,000 g for 3 h at 4°C. The endosome-enriched fraction containing vesicles positive for markers of the early and recycling endosomes at the 25%/35% sucrose interface was collected, diluted threefold with PBS, and spun at 108,000 g for 30 min at 4°C. Pelleted endosomes were resuspended in 0.1% BSA/PBS. Rabbit anti-Rab11a, anti-Rab11b, Na-K-ATPase, or a nonspecific rabbit IgG was added to apoertioned samples and incubated with the isolated endosomes overnight at 4°C with rotation. During this period, sheep anti-rabbit magnetic Dynabeads (Invitrogen) were washed with 1% BSA/PBS three times and incubated with 1 ml 1% BSA/PBS overnight at 4°C. Following washing, the beads were recovered with a magnet and resuspended in 50 μl of 1% BSA/PBS. The blocked and washed beads were then added to the samples and incubated with each of the antibody-endosome fractions for 6 h at 4°C with rotation. The bead antibody-endosome complexes were collected with a magnet, washed twice with 1% BSA/PBS, once with 0.1% BSA/PBS, and then once with PBS. Laemmli sample buffer was added to the immunoisolated endosomes, and samples were resolved on 6-18% SDS-PAGE, transferred to polyvinylidene difluoride (1 h at 100 V), and blotted for proteins of interest.

**Short-circuit current and membrane capacitance recordings.** Cells cultured on filter supports were mounted in modified Ussing chambers (manufactured by W. Van Driesche, Leuven, Belgium) using our manufacturer’s instructions as described previously (O’Brien Center, Vector Core, University of Pittsburgh, PA) (62).

**Immunofluorescence labeling.** Routine immunofluorescent labeling of samples was performed as follows. Unless described otherwise, all steps were performed at 4°C. Polarized, filter-grown mpkCCD cells were washed three times with PBS containing 0.1 mM CaCl2 and 1 mM MgCl2 (PBS + CM) and fixed with 4% paraformaldehyde in PBS + CM for 30 min. Following three PBS + CM washes, the cells were permeabilized with 0.1% Triton X-100 and 0.1% NP-40 in PBS + CM for 20 min for samples requiring antibody labeling. Cells were labeled in blocking buffer consisting of 10% normal goat serum, 10% dry nonfat milk, and 0.05% Triton X-100 in PBS + CM overnight at 4°C. Unbound primary antibody was removed by four washes with PBS + CM. Primary antibodies were labeled with corresponding fluorescence-conjugated secondary antibodies in blocking buffer for 2 h at 37°C. For cells expressing GFP-tagged constructs, no antibody labeling was required and following counterstaining with 4,6-dimino-2-phenylindole cells were washed again and mounted on coverslips in fluoromount-G (Southern Biologicals, Birmingham, AL) for imaging. Images were captured using an Olympus IX81 fluorescence microscope (Olympus, Center Valley, PA) fitted with a DSU spinning disk and 300-W fluorescent light source using either a ×60, 1.4-numerical aperture (NA) oil or ×20, 0.45-NA long working distance air objective. Single fluorescent images were captured using a Retiga cooled CCD camera (QImaging, Surrey, BC) at 1.024 × 1.024 resolution using SlideBook (Olympus). Linear adjustments of brightness and contrast were made offline in MetaMorph ( Molecular Devices, Downingtown, PA).

For dual-label colocalization studies, filter-grown cells were fixed and processed using a pH-shift protocol as described previously (2). With the antibodies of the same species were used, a sequential staining protocol (adapted from one provided by Jackson Immunoresearch) was employed. Following fixation, unreacted paraformaldehyde was quenched with PBS containing 20 mM glycine, pH 8.0 and 75 mM NH4Cl for 10 min at room temperature. Cells were then incubated for 30 min at room temperature in a blocking buffer containing 0.025% (wt/vol) saponin and 8.5 mg/ml of fish skin gelatin dissolved in PBS. The cells were incubated with primary antibody, diluted in blocking buffer for 16 h at 4°C, and then washed three times with blocking buffer for 5 min each. The samples were then incubated for 16 h at 4°C with 13 μg/ml goat-anti-rabbit IgG Fab fragments, washed three times with blocking solution, and then incubated for 1 h at room temperature with secondary antibody (donkey anti-goat Cy3 diluted 1:3,000). Following washes with blocking buffer and PBS, the cells were postfixed with 4% paraformaldehyde in 100 mM sodium cacodylate (pH 7.4) for 5 min at room temperature. The unreacted paraformaldehyde was quenched for 5 min at room temperature with the quenching buffer described above. The cells were incubated with blocking solution for 30 min at room temperature and then incubated with the next rabbit primary antibody for 2 h at room temperature. After three 5-min washes with blocking buffer, the cells were incubated with a tertiary antibody (donkey anti-rabbit FITC, diluted 1:200) for 1 h at room temperature. The cells were rinsed with blocking buffer and then PBS, post-fixed, and then mounted as described previously (2). In control reactions, the Fab fragments or individual primary antibodies were left out of the incubations (not shown).

Imaging was performed using a TCS-SL confocal microscope (Leica, Deerfield, IL) equipped with argon, green helium-neon, and
red helium-neon lasers. Images were acquired using a ×100 1.4-NA oil objective. Photomultipliers were set to 600–900 V and zoom at ×4.0. Images were collected every 0.30 μm and averaged four times. All pixel values fell within the 8-bit range of the captured image files. The images (512 × 512 pixels) were saved in a TIFF format, contrast was corrected in Photoshop, and electronic files were generated in Adobe Illustrator.

Stacks of dual-labeled confocal sections were imported into Velocity (PerkinElmer, Waltham, MA), background noise was removed using the fine (3 × 3) median noise reduction filter, and a scatter plot of voxel intensities for each of the markers was generated using the colocalization function. The images were thresholded using a fixed value of 15, and Mander colocalization coefficients for each of two markers (Mx and My, respectively) were calculated for the entire three-dimensional image using the following equations (1)

\[ M_x = \frac{\sum X_{x,\text{colocalized}}}{\sum X_x} \]

\[ M_y = \frac{\sum Y_{y,\text{colocalized}}}{\sum Y_y} \]

where \( X_x \) is equal to the intensity of marker X at a given voxel and \( X_{x,\text{colocalized}} = X_x \) if the associated intensity of the other marker (\( Y_y \)) is above the threshold value and therefore colocalizes. When \( X_{x,\text{colocalized}} = 0 \), it indicates that \( Y_y \) is below the threshold value and does not colocalize. \( Y_y \) and \( Y_{y,\text{colocalized}} \) are similarly defined. An \( M_x \) or \( M_y \) value of 1.0 indicates 100% colocalization, while a value of 0.0 indicates no colocalization. The values for \( M_x \) and \( M_y \) can be similar or not, as one marker may have a broader distribution than the other in the sampled region of the tissue.

siRNA. To knock down the expression of Rab11a or Rab11b, siRNAs specific for each mouse isoform (4 constructs/isosform) were commercially obtained (Dharmacon/Thermo Fisher Scientific, On-Target Plus) and introduced into the mpkCCD cells using Lipofectamine 2000 at a concentration of 50 nM as described previously (10). The target sequences for Rab11a and Rab11b were as follows: 1) GUACAGGGCUAUAACGUCU, UAAGAGGUAUUAACGUCU, GGGCGAGCGAGUACGACUA, and UAACAGAGAUACCCGCU for Rab11a; and, 2) GGCGAGCGGAUCAGACUU, GCAUUCAGGGACGCAGCA, UGCAUAAGAGACGGGAGGA, and CAUUCUGACAGAUAGGGA for Rab11b. Cells were seeded onto filter supports and allowed to polarize over 72 h before use in electrophysiological experiments. Following Isc and Cx measurements, the cells were harvested from the filter supports in lysis buffer containing protease inhibitors (as above) and proteins were resolved by SDS-PAGE to determine the extent of protein knockdown.

Surface biotinylation. To demonstrate the change in ENaC surface expression following siRNA knockdown of Rab11 isoforms, we performed surface biotinylation using a modified protocol similar to the approach we described before in these cells (11). Basically, cells were transfected with siRNA as described above and seeded onto 6.5-mm-diameter filter inserts (Transwell, Corning Costar). There were three groups of cells: control (nontargeting) siRNA, Rab11a, and Rab11b siRNA-transfected cells. A total of 12 filters were used for each group and pooled following biotinylation to obtain 1 sample. The inserts were first washed five times with ice-cold PBS containing MgCl2 and CaCl2 to remove medium containing FBS. The cells were biotinylated at 4°C in borate buffer (85 mM NaCl, 4 mM KCl, 15 mM Na2B4O7, 375 μg biotin at pH 9) on the apical surface with the basolateral side of the monolayer bathed in medium containing FBS to prevent basolateral biotinylation. After 20 min, basolateral and apical sides were aspirated and medium containing FBS was placed on the cells to quench the signal. Monolayers were washed five times with ice-cold PBS with agitation, and the cells were harvested by scraping in PBS using a 200-μl pipette tip. The cell homogenate was obtained by lysing cells in lysis buffer (see above) and then centrifugation for 5 min at 5,000 rpm. The cell homogenate was assayed for protein concentration, and a small (20 μl) aliquot of the total lysate was removed to be used as whole cell lysate to probe for intracellular proteins and as a loading control. For the biotinylated sample, 300 μg of protein was incubated with 150 μl avidin bead slurry as previously described (11). Samples were heated to 95°C for 8 min and separated on a 10% SDS-PAGE. Samples were transferred to nitrocellulose membranes (Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA) following the manufacturer’s protocol. Nitrocellulose was blocked in 5% skim milk constituted in PBS for 3 h. The membrane was transferred to 1% skim milk-PBS containing antibodies (1:1,000 α-ENaC, 1:250 β,γ-ENaC) at 4°C overnight. Following antibody incubation, incubation blots were washed four times in PBS. Horseradish peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD) were diluted 1:5,000 in 1% skim milk-PBS. Membranes were incubated with secondary antibody for 1 h at room temperature. The membrane was washed twice for 30 s in PBS followed by one 15-min wash and four 5-min washes. Reactive proteins were visualized with enhanced chemiluminescence (PerkinElmer Life Sciences, Wellesley, MA). Western blots were quantified after a digital capture (scanning) using Photoshop CS. Band intensities were normalized to actin (WCL), corrected for background, and expressed as a percentage of the control siRNA levels (∼n = 2).

Statistics. All data were analyzed using SigmaPlot (Systat, Chicago, IL). Summarized data were evaluated for normality and equal variance, and t-tests were carried out to determine whether differences were statistically different from each other. For any difference in the mean values, P < 0.05 was considered significantly different.

RESULTS

Characterizing a new anti-mouse α-ENaC antibody. Due to previous difficulties in obtaining reliable ENaC antibodies for use with mouse tissue, we had a purified rabbit polyclonal antibody manufactured to specifically recognize the mouse α-ENaC subunit. Characterization of this antibody involved transiently overexpressing tagged versions of mouse ENaC in FRT cells and running the whole cell lysate on SDS-PAGE. The antibody specifically identified the full-length form of the mouse α-ENaC subunit and did not cross react with either β- or γ-ENaC (Fig. 1A). In addition, the shift in apparent molecular weight was observed in the GFP-tagged ENaC construct compared with the HA-tagged version of the expression plasmid. Along with specific recognition of α-ENaC by Western blotting, the antibody could also be used for immunofluorescent labeling (Fig. 1B). Preimmune bleeds and peptide competition experiments also verified the specificity of this new ENaC antibody.

ENaC shows greater colocalization with Rab11b than Rab11a. Previous studies have identified the expression of Rab11 in rat kidney collecting duct tissue (4). However, there is little data concerning the isoform-specific expression of Rab11 in kidney cells and tissues. There is also a lack of information about whether ENaC is localized in a Rab11 isoform-selective manner in kidney epithelial cells. Using specific Rab11a and Rab11b antibodies (see Fig. 5), we confirmed that both isoforms were expressed in mpkCCD cells (Fig. 2). Each protein showed a punctate, vesicular distribution, and most of the punctae concentrated at the apical pole of the cells; however, punctae were also observed along the lateral margins of the cell (Fig. 2, B, E, and H). Consistent with a previous analysis in Madin-Darby canine kidney and gastric parietal cells (13, 41), we observed only a small degree of...
colocalization between these two isoforms (colocalization coefficient of Rab11a vs. Rab11b = 0.16; Rab11b vs. Rab11a = 0.22; Table 1; Fig. 2, G–I).

Next, we used an α-ENaC antibody (Fig. 1) and a sequential labeling protocol to examine the localization of ENaC with both Rab11 isoforms. Like Rab11, α-ENaC was found in small punctate vesicular elements that concentrated at the apical pole of the cells. We observed that α-ENaC colocalized with Rab11a (colocalization coefficient of 0.28; Table 1) (Fig. 2, A–C); however, a significantly greater pool of α-ENaC colocalized with Rab11b (colocalization coefficient of 0.51; Table 1; P < 0.05) (Fig. 2, D–F). These results indicate that mpkCCD cells express both Rab11 isoforms and that ENaC may be preferentially associated with the Rab11b pool of vesicles.

ENaC is present in immunoisolated Rab11 vesicles. A magnetic bead immunoisolation technique was employed to verify that ENaC was colocalized in Rab11-positive vesicles. Unlike immunoprecipitation techniques, immunoisolation does not require that proteins physically interact. Endosomal vesicles were isolated from filter-grown mpkCCD cells by discontinuous sucrose gradient centrifugation. This technique has been employed previously by us to isolate the chloride channel CFTR in Rab11-positive vesicles (62). The isolated vesicles were incubated with antibodies that recognize either Rab11a, Rab11b, or control antibodies and separated magnetically by incubation with secondary antibodies attached to magnetic beads. The α-subunit of Na-K-ATPase and nonspecific preimmune IgG were used as negative controls. Isolated samples were resolved on gradient SDS-PAGE and nonspecific primary antibody showed little detectable amounts of Rab11b in the Rab11a-immunoisolated endosomes and vice versa (Fig. 3). This technique also confirmed β-ENaC was present in both Rab11a- and Rab11b-positive endosomes. Control immunoisolations using the Na-K-ATPase or control antibody showed little detectable amounts of Rab11 or ENaC. These studies confirm that ENaC is associated with both Rab11a- and Rab11b-positive pools of endosomes.

DN-Rab11 expression reduces ENaC currents and prevents ENaC recycling. As indicated in the introduction, mutations in Rab-GTPases are able to lock the proteins into either the active, GTP-bound, or inactive, GDP-bound state. Following transient transfection of wild-type (wt) Rab11a and wtRab11b or the DN-Rab11 forms, cells were seeded onto filter supports to record ENaC-mediated ISC in Ussing chambers. A significant reduction in cAMP-stimulated ENaC current was observed in cells transiently transfected with DN-Rab11b (Fig. 4). However, no significant change in ENaC currents was observed in cells overexpressing Rab11a-DN, wt-Rab11a, or wtRab11b constructs. The lack of a significant response following DN-Rab11a overexpression could be due to low transfection efficiency (~50%), and we therefore developed adenoviral DN-Rab11 constructs to permit a more acute and titratable expression of the DN-Rab11 in mpkCCD cells.

Table 1. Colocalization coefficients

<table>
<thead>
<tr>
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<th>Means ± SE (n)</th>
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<tbody>
<tr>
<td>ENaC vs. Rab11a</td>
<td>0.281 ± 0.070 (5)</td>
</tr>
<tr>
<td>Rab11a vs. ENaC</td>
<td>0.213 ± 0.010 (5)</td>
</tr>
<tr>
<td>ENaC vs. Rab11b</td>
<td>0.516 ± 0.043 (5)</td>
</tr>
<tr>
<td>Rab11b vs. ENaC</td>
<td>0.296 ± 0.031 (5)</td>
</tr>
<tr>
<td>Rab11a vs. Rab11b</td>
<td>0.160 ± 0.020 (3)</td>
</tr>
<tr>
<td>Rab11b vs. Rab11a</td>
<td>0.228 ± 0.012 (3)</td>
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The proportion of colocalized immunofluorescent signal for labeled Rab11a, Rab11b, and epithelial sodium channel (ENaC) or Rab11a and Rab11b is presented as a mean of >3 different experiments (n). A significantly (*) greater proportion of ENaC signal was localized with Rab11b than Rab11a (P < 0.05).
Representative fluorescent images of mpkCCD cells expressing GFP-DN-Rab11 constructs are presented in Fig. 5. Addition of the GFP tag increased the apparent molecular weight of the expressed mutant Rab11 proteins, making them readily detectable over endogenous Rab11 isoforms by Western blotting. The viral particle load and protein expression were determined for each virus to ensure comparable levels of overexpression of Rab11 were achieved (95% transfection efficiency). Expression of GFP-Rab11a or GFP-Rab11b resulted in a diffuse cytoplasmic localization of the GFP-tagged Rab11.

Electrophysiological recordings were performed 24 h after viral infection to assess the impact on ENaC-mediated current and changes in membrane capacitance (C\text{T}) following cAMP stimulation. We demonstrated previously that C\text{T} recordings constitute a reliable readout of membrane surface area (9). The changes in C\text{T} in the mpkCCD cells are due to delivery or removal of membrane vesicles during vesicle-trafficking events at the apical surface. When vesicles are delivered to and fuse with the apical membrane during exocytosis, there is a corresponding increase in C\text{T}. Conversely, when vesicles are endocytically retrieved from the membrane there is a decline membrane surface area. A change in C\text{T} is apparent under non-steady-state conditions, for example, when a pool of ENaC-containing vesicles are induced to fuse with the apical surface following cAMP stimulation. By repeatedly simulating cells with cAMP, we can induce rounds of insertion and retrieval to monitor vesicle-trafficking events.

Sample traces of both I\textSC and C\text{T} measurements are presented in Fig. 6. Expression of DN-Rab11b produced a significant reduction in stimulated I\textSC, while the other constructs had no significant impact compared with GFP-transfected controls (n = 5, *P < 0.05).

Fig. 3. ENaC localized in immunoisolated Rab11-positive vesicles. Following isolation of Rab11a- and Rab11b-specific vesicles, samples were resolved by SDS-PAGE. As controls, the α-subunit of the Na-K-ATPase (localized to the basolateral membrane) and nonspecific IgG antibodies were used to verify the specificity of ENaC isolation. Blots were sequentially probed using Rab11a, Rab11b, β-ENaC, and the Na-K-ATPase antibodies. ENaC was localized to both Rab11a- and Rab11b-positive vesicles, but was not detected in the control isolations. A portion of Rab11a was detected in the Rab11b-positive samples and vice versa. Blots are representative of 2 similar experiments.

Fig. 4. Amiloride-sensitive short-circuit current (I\textSC) in Rab11-transfected cells. Rab11 plasmids were transient transfected into mpkCCD cells, which were seeded onto permeable filter supports for electrophysiological measurements. The forskolin-stimulated, amiloride-sensitive I\textSC in either Rab11-WT or dominant negative (DN) constructs were normalized to control I\textSC values. Overexpression of DN-Rab11b produced a significant reduction in stimulated I\textSC, while the other constructs had no significant impact compared with GFP-transfected controls (n = 5, *P < 0.05).

Fig. 5. Adenoviral overexpression of Rab11. A: to reliably overexpress mutants of Rab11, mpkCCD cells were infected using adenoviral constructs containing either DN-Rab11a-GFP, DN-Rab11b-GFP, or GFP alone as a control. Expression of each Rab11 isoform was determined by Western blotting using isoform-specific antibodies (or an anti-GFP antibody as a control) to demonstrate overexpression of the DN-Rab11 constructs. B: as the constructs were GFP tagged, it was possible to monitor the level of overexpression by live cell fluorescent microscopy. An example of adenoviral-infected mpkCCD cells cultured on filter supports is presented for each of the constructs. Scale bars = 50 μm.
tion was also significantly reduced. The increase in $C_T$ following cAMP stimulation is a measure of the number of vesicles that traffic to the surface in response to this agonist. The $ΔC_T$ change in control cells (GFP alone infected) was 0.12 ± 0.013 μF/cm² compared with DN-Rab11a with 0.08 ± 0.008 μF/cm² and DN-Rab11b with a $ΔC_T$ of 0.03 ± 0.005 μF/cm² ($n = 9$, $P < 0.01$ for control vs. DN-Rab11a or -b). The reduction of the cAMP response was likely due to an inability of the DN-Rab11-expressing cells to deliver ENaC-containing vesicles to the surface.

It is possible to estimate the number of vesicles fusing with the apical surface that result in the observed $C_T$ increase (see Ref. 5 for details). If the specific capacitance of a biological membrane is assumed to be 1 μF/cm², then we can estimate a value of $9.6 \times 10^7$ vesicles/cm² are exocytosed following cAMP stimulation in control mpkCCD cells and $2.4 \times 10^7$/cm² for DN-Rab11b-expressing cells. Taking this one step further by estimating the cell density for mpkCCD cells cultured on filter supports ($\sim 2.5 \times 10^3$/cm²), it equates to $\sim 375$ vesicles/cell in control cells compared with $\sim 95$ vesicles/cell in the DN-Rab11b-expressing cells. Even without the final estimation of vesicles per cell, there is approximately four times the number of vesicles fusing with the apical membrane in control cells compared with the DN-Rab11b-expressing cells. While the expression of DN-Rab11a reduced both $I_{SC}$ and a $C_T$, it was not to the same extent as DN-Rab11b when protein levels of the DN constructs of the two isoforms were similarly expressed.

**Rab11 knockdown.** With expression of DN-Rab11 resulting in a significant reduction in ENaC-mediated Na⁺ transport, there was still the possibility of a nonspecific, off-target effect of DN-Rab11b expression causing the reduction in ENaC currents. Similarly, as the two isoforms of Rab11 share significant homology, it is possible that the high expression of DN-Rab11a may nonspecifically interact with GEFs that would normally associate with Rab11b. Therefore, to address the roles of each Rab11 isoform in ENaC regulation directly, the protein levels Rab11a and Rab11b were specifically knocked down using a siRNA approach. The isoform-specific knockdown of Rab11b was confirmed by Western blotting with a >80% decrease in protein expression obtained for each isoform (Fig. 7). No off-target effects were noted with control knockdown of GAPDH or a scrambled siRNA construct. Following siRNA transfection, cells were seeded onto filter supports and allowed to polarize for 48 h, before being mounted in Ussing chambers for electrophysiological measurements. The basal and forskolin-stimulated amiloride-sensitive $I_{SC}$ was significantly reduced in the Rab11b knockdown cells, but not reduced in cells with Rab11a knockdown compared with controls (Fig. 7B). In summary, these results indicate that...
both steady-state ENaC surface expression and cAMP-mediated ENaC trafficking are likely regulated by Rab11b, and not Rab11a.

ENaC surface biotinylation. To demonstrate that the reduction in measured $I_{SC}$ following Rab11b knockdown was due to a change in channel surface density, we performed surface biotinylation on filter-cultured mpkCCD cells where each Rab11 isoform was knocked down separately (as above). There was a small (~30%) reduction in surface expression of each ENaC subunit following Rab11a knockdown and a greater loss of surface ENaC expression (~80%) following Rab11b knockdown. These data indicate that the loss of ENaC current with Rab11b knockdown was likely the result of inability of the mpkCCD cells to deliver ENaC to the apical surface, and not due to changes in channel activity. The reduction in surface expression was also not due to a loss of whole-cell ENaC expression as evident from the quantification of whole cell levels presented in Fig. 8B.

DISCUSSION

Following clathrin- and epsin-dependent apical endocytosis, ENaC can be recycled constitutively or via a regulated pathway that is stimulated by mediators that increase cAMP production (8–10). The nature of these recycling pathways are unknown, but it is likely that ENaC recycling involves the passage through one or more endocytic compartments in a process facilitated by members of the Rab family of small GTPases. Among the specific Rabs that are localized to endocytic compartments, Rab11 is a key regulator of apical recycling pathways in polarized epithelial cells (13, 16, 38, 41, 62). It is known to facilitate the recycling of a number of receptors and transporters including IK channels, CFTR, Kv1.5, aquaporin 2, CIC-2, and ENaC (26, 38, 56, 62, 65). Our previous work identified delivery of ENaC into early endosomal compartments via clathrin-mediated endocytosis following its removal from the apical membrane (68). For ENaC to be returned to the apical surface following endocytosis, it most likely passes through several vesicle compartments in a process facilitated by members of the Rab family of small GTPases.

The current results, and our recent discovery of a role for Rab11b in CFTR recycling (62), indicate that Rab11b may have an important role in the regulation of epithelial channel recycling and surface expression. We confirmed the role for Rab11b in ENaC surface delivery by several investigations. First, we observed that there was a large degree of colocalization between ENaC and Rab11b in immunolocalization studies. Second, ENaC was found in immunosolated Rab11b endosomes. Third, expression of DN-Rab11b dramatically inhibited ENaC activity, which capacitance measurements indicate results from a large decrease in vesicle exocytosis and a reduction in surface expression of ENaC. Fourth, downregulation of Rab11b expression by siRNA resulted in a dramatic loss of ENaC activity. Finally, surface biotinylation confirmed the loss of ENaC surface expression following a reduction in Rab11b levels. It is unclear why there was little loss of ENaC activity or surface expression when Rab11a expression was decreased by siRNA, as a previous study demonstrated a role for Rab11a in exocytosis of ENaC in CHO cells (38). Expression of the DN-Rab11a construct produced an intermediate reduction in ENaC current and $C_T$ compared with expression of DN-Rab11b.

Fig. 8. Surface biotinylation demonstrates reduced ENaC expression after Rab11b knockdown. A: surface biotinylation was performed on filter-cultured mpkCCD cells transfected with control, Rab11a, or Rab11b siRNA. Apical surface expression of the 3 ENaC subunits is presented in the top blots, with an actin control to demonstrate the specificity of the biotin labeling. Whole-cell lysate (WCL) blots are presented at the bottom to probe for whole cell levels of ENaC and Rab11. B: quantification of band intensities for $n > 2$ experiments are presented for all proteins. *Significant reduction ($P < 0.01$) in expression compared with control siRNA cells.
The open question that remains, however, is why ENaC is found in both Rab11a and Rab11b endosomes. One possibility is that ENaC may sequentially pass through both Rab11a and Rab11b compartments at some point in its lifetime. Alternatively, there may be distinct pools of ENaC that are recycling through different populations of endosomes. An additional possibility is that the Rab11a- and Rab11b-localized channels have different fates. For example, ENaC in Rab11b endosomes may recycle relatively rapidly, whereas that in Rab11a endosomes may be undergoing slow recycling to the Golgi. Intracellular Golgi network, a process that is known to be regulated by Rab11a (44, 69). We have also not explored the possibility that the state of proteolytic cleavage may alter the fate of recycling ENaC.

In addition to Rab11, prior studies have linked Rab4 and Rab27 to ENaC regulation (3, 54, 58), demonstrating that the Rab GTPases are important mediators of ENaC trafficking and that trafficking plays a key role in determining ENaC density at the membrane surface. Our data further establish Rab11b as an essential component of the trafficking pathway regulating ENaC surface density in mpkCCD cells. Further investigation will be required to define the conditions under which the channel enters each Rab11-positive compartment, and the role of each in determining ENaC surface expression.

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DISCLOSURES

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