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

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Butterworth MB, Edinger RS, Silvis MR, Gallo LI, Liang X, Apodaca G, Fizzell RA, Johnson JP. Rab11b regulates the trafficking and recycling of the epithelial sodium channel (ENaC). Am J Physiol Renal Physiol 302: F581–F590, 2012. First published November 30, 2011; doi:10.1152/ajprenal.00304.2011.—Expression of the epithelial sodium channel (ENaC) at the apical membrane of cortical collecting duct (CCD) principal cells is modulated by regulated trafficking mediated by vesicle insertion and retrieval. Small GTPases are known to facilitate vesicle trafficking, recycling, and membrane fusion events; however, little is known about the specific Rab family members that modify ENaC surface density. Using a mouse CCD cell line that endogenously expresses ENaC (mpkCCD), the channel was localized to both Rab11a- and Rab11b-positive endosomes by immunosolulation and confocal fluorescent microscopy. Expression of a dominant negative (DN) form of Rab11a or Rab11b significantly reduced the basal and cAMP-stimulated ENaC-dependent sodium (Na+) transport. The greatest reduction in Na+ transport was observed with the expression of DN-Rab11b. Furthermore, small interfering RNA-mediated knockdown of each Rab11 isoform demonstrated the requirement for Rab11b in ENaC surface expression. These data indicate that Rab11b, and to a lesser extent Rab11a, is involved in establishing the constitutive and cAMP-stimulated Na+ transport in mpkCCD cells.

small GTPase; siRNA; dominant-negative Rab

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 underlying 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 EE1-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 EE1 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 Rab 4, 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).
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 regulation of ENaC ectopically expressed in Chinese hamster ovary (CHO) cells (38), no studies have reported a role for Rab11b in apical recycling of ENaC, and we sought to clarify the involvement of each Rab11 isoform in a cell line in which ENaC is endogenously expressed and regulated (62, 65).

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 α1-1a-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 phallolidin-Alexa 633 (Invitrogen, Carlsbad, CA).

ENaC antibody characterization. An affinity-purified rabbit polyclonal antibody was raised against α-ENaC using a 14-amino 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)-ENaC and double-tagged hemagglutinin (HA)-ENaC-V5 were kindly provided by Drs. J. Stockand, UTHSCSA, San Antonio, TX and T. Kleyman (Renal-Electrolyte Division, School of Medicine, University of Pittsburgh), respectively, and have been previously characterized (31, 64). FRT cells were transfected with ENaC using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. As a control, an eYFP-membrane reporter plasmid was used (Clontech, Mountain View, CA). Cell lysates of transfected and control FRT cells were obtained (as described below), and proteins were resolved by SDS-PAGE to test the specificity of the antibody in standard Western blots. In addition, mpkCCD cells cultured on filters were prepared for immunofluorescent imaging, biotinylation, and Western blotting (see below). Controls included incubation of the primary antibody with the immunizing peptide (1 mg/ml) for 1 h at 37°C before incubation with the sample and the use of preimmune serum to check for a specific signal (Fig. 1). Bands observed on Western blots using the anti-α-ENaC antibody were verified by incubation with an anti-HA antibody against the epitope-tagged α-ENaC. We were unable to detect cleaved forms of α-ENaC in these overexpressing FRT cells, and Western blotting with the anti-ENaC antibody or the HA-tagged form of the channel.

Cell culture. The mpkCCD-c14 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 mM sodium selenate, 5 mg/ml transferrin, 2 mM glutamine, 50 mM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 g/ml insulin, 20 mM α-glucose, 2% vol/vol FCS, and 20 mM HEPES (Invitrogen, Sigma), pH 7.4, at 37°C in 5% CO\(_2\)/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-cm\(^2\) surface area; Transwell, Corning, Lowell, MA). Cells were cultured for at least 7 days in 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-cm\(^2\) 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 "chopsstick" 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.

**Immunoisolation of Rab11-positive endosomes.** The immunoisolation 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 polycryl 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 non-specific rabbit IgG was added to apportioned 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. Lasermi 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 (Harvard Apparatus, Holliston, MA), and the cultures were continuously short circuited with an automatic voltage clamp in a system that permitted simultaneous detection of short-circuit current (Isc) and total membrane capacitance (CT) to be performed (designed and manufactured by W. Van Driesche, Leuven, Belgium), using our previously described methods (9–11). The bathing Ringer solution was composed of 120 mM NaCl, 25 mM NaHCO3, 3.3 mM KH2PO4, 0.8 mM K2HPO4, 1.2 mM MgCl2, 1.2 mM CaCl2, and 10 mM glucose. Chambers were constantly gassed with a mixture of 95% O2-5% CO2 at 37°C, which maintained the pH at 7.4. Chamber washes were carried out by a fivefold volume exchange (25–30 ml) with 37°C Ringer solution to wash out each chamber’s basolateral bathing solution. As the cells were sensitive to changes in pressure, flow, and temperature, the chamber solutions were only exchanged during wash periods with no flow at steady state. A typical stimulation protocol involved the addition of 10 μM forskolin (Fisher Scientific, Pittsburgh, PA) basolaterally, which produced a maximum Isc stimulation after 20–30 min; forskolin was washed from the basolateral side of the chamber, and current declined back to basal levels within 30 min. To determine the net Na+ transport through ENaC, 10 μM amiloride (Sigma) was added to the apical cell surface at the end of each experiment.

**DNA constructs: Rab11a- and b-S25N adenoviruses.** Generation of the adenoviral constructs was recently described in detail (62). GFP-tagged Rab11a and Rab11b were TOPO-cloned into the pCR2.1 vector (Invitrogen) and then sequenced using M13 primers (Invitrogen). The S25N and Q70L point mutations were introduced into the wild-type GFP-Rab11b construct using a Quick-Change Site-directed Mutagenesis kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. All constructs were sequenced before use. Recombinant adenovirus-expressing GFP-tagged Rab11a-S25N (pAdTetGFP-Rab11a-S25N) and adenovirus-expressing GFP-Rab11b-S25N was created using the Vira-Power Adenoviral Expression System (Invitrogen) per the 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-phenylinodole cells were washed again and mounted on coverslips in fluoromount-G (SouthernBiotech, Birmingham, AL) for imaging. Images were captured using an Olympus IX81 fluorescent 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 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). When 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, Dearfield, 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×. 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)

\[
M_x = \frac{\sum X_{i, \text{colocalized}}}{\sum X_i}
\]

\[
M_y = \frac{\sum Y_{i, \text{colocalized}}}{\sum Y_i}
\]

where \(X_i\) is equal to the intensity of marker X at a given voxel and \(X_{i, \text{colocalized}}\) is the adjusted intensity of the other marker (Y) if above the threshold value and therefore colocalizes. When \(X_{i, \text{colocalized}} = 0\), it indicates that Y is below the threshold value and does not colocalize. \(Y_i\) and \(Y_{i, \text{colocalized}}\) 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.

siRNA. To knock down the expression of Rab11a or Rab11b, siRNAs specific for each mouse isoform (4 constructs/isoform) 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) GUACAGGC-GUAUAACGUCU, UAAGAGGAUAAUACGCUA, GGCGAGAC-GAGUAAGCAGUA, and UAACAGAGAUACCCGCAU for Rab11a; and, 2) GGCGAGCGAGUAGCAUAU, GCAUUCAGGAGAGGC-A, GGAUAAGGAGACAAGGGA, and GACUCUGACAG-GAUAGGGA for Rab11b. Cells were seeded onto filter supports and allowed to polarize over 72 h before use in electrophysiological experiments. Following Incucyte and Cytoscape 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 filters were first washed five times with ice-cold PBS containing Mg2+ and Ca2+ 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 digital capture (scanning) using Adobe 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 cells (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 immune IgG were used as negative controls. Isolated samples were resolved on gradient SDS-PAGE and nonspecific immunoblotting probed and blotted with antibodies against β-ENaC, Rab11a, Rab11b, or the Na-K-ATPase. In agreement with the immunofluorescent studies presented above, we observed small 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 immunosolations 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 ($C_T$) following cAMP stimulation. We demonstrated previously that $C_T$ recordings constitute a reliable readout of membrane surface area (9). The changes in $C_T$ 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 $C_T$. Conversely, when vesicles are endocytically retrieved from the membrane there is a decline in membrane surface area. A change in $C_T$ 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.

Overexpression of DN-Rab11b produced a significant reduction in stimulated $I_{SC}$, while the other constructs had no significant impact compared with GFP-transfected controls ($n = 5$, *$P < 0.05$).

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**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 ($I_{SC}$) 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 $I_{SC}$ in either Rab11-WT or dominant negative (DN) constructs were normalized to control $I_{SC}$ values. Overexpression of DN-Rab11 produced a significant reduction in stimulated $I_{SC}$, 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.
The expression of DN-Rab11a reduced both cell compared with the DN-Rab11b-expressing cells. While the number of vesicles fusing with the apical membrane in control vesicles per cell, there is approximately four times the DN-Rab11b-expressing cells. Even without the final estimation cell in control cells compared with control of vesicles in the apical membrane is assumed to be 1 \( \mu F/cm^2 \) (Ref. 5 for details). If the specific capacitance of a biological membrane is 1 \( \mu F/cm^2 \) compared with DN-Rab11a with 0.08 \( \pm 0.008 \) \( \mu F/cm^2 \) and DN-Rab11b with a \( \Delta C_F \) of 0.03 \( \pm 0.005 \) \( \mu F/cm^2 \) (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 \( C_F \) increase (see Ref. 5 for details). If the specific capacitance of a biological membrane is assumed to be 1 \( \mu F/cm^2 \), then we can estimate a value of 9.6 \( \times 10^7 \) vesicles/cm\(^2\) are exocytosed following cAMP stimulation in control mpkCCD cells and 2.4 \( \times 10^7\) vesicles/cm\(^2\) for DN-Rab11b-expressing cells. Taking this one step further by estimating the cell density for mpkCCD cells cultured on filter supports \((~2.5 \times 10^6/cm^2)\), it equates to ~375 vesicles/cell in control cells compared with ~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 \( C_F \), it was not to the same extent as DN-Rab11b when protein levels of the ENaC 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 and membrane trafficking, the expression of ENaC-containing vesicle exocytosis. The number of observations for each group is the same as in B. #Significant difference (\( P < 0.01 \)) from GFP control.

**Fig. 6.** DN-Rab11 expression reduces ENaC-mediated \( Na^+ \) transport and membrane trafficking. A: representative \( I_SC \) recordings from mpkCCD cells expressing either GFP (black trace) as a control or DN-Rab11b-GFP (grey trace). Cells were stimulated with 10 \( \mu M \) forskolin (Forsk) during the indicated periods (bar above trace) to induce ENaC trafficking to the apical membrane. At the end of the recording, the measured \( I_SC \) was inhibited with 10 \( \mu M \) amiloride (Amil) to block ENaC transport. Both the basal and cAMP-stimulated ENaC-dependent \( I_SC \) were significantly inhibited by overexpression of DN-Rab11 b compared with control.

**Fig. 7.** Rab11b knockdown reduces ENaC-mediated \( Na^+ \) transport. By 10.22.03.247 on November 7, 2016 http://ajprenal.physiology.org/Downloaded from AJP-Renal Physiol • doi:10.1152/ajprenal.00304.2011 • www.ajprenal.org
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, ClC-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 immunoisolated 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

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 mnpCCD 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
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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