The carboxy terminus of the α-subunit of the amiloride-sensitive epithelial sodium channel (ENaC) binds to F-actin

Christopher Mazzochi1, James K. Bubien2, Peter R. Smith2 and Dale J. Benos2

From the 1Department of Cell Biology, 2Department of Physiology and Biophysics, The University of Alabama at Birmingham, Birmingham, Alabama 35294.

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Address correspondence to: Dale J. Benos, Dept. of Physiology and Biophysics, MCLM 704, University of Alabama at Birmingham, 1918 University Blvd, Birmingham, Alabama 35294-0005, Tel. (205) 934-6220; Fax (205) 934-2377; E-mail: benos@physiology.uab.edu

The activity of the amiloride-sensitive epithelial sodium channel (ENaC) is modulated by F-actin. However, it is unknown if there is a direct interaction between α-ENaC and actin. We have investigated the hypothesis that the actin cytoskeleton directly binds to the carboxy terminus of α-ENaC using a combination of confocal microscopy, co-immunoprecipitation, and protein binding studies. Confocal microscopy of Madin-Darby canine kidney cell monolayers (MDCK) stably transfected with wild type, rat isoforms of alpha, beta, and gamma ENaC revealed co-localization of α-ENaC with the cortical F-actin cytoskeleton both at the apical membrane and within the subapical cytoplasm. F-actin was found to co-immunoprecipitate with α-ENaC from whole cell lysates of this cell line. Gel overlay assays demonstrated that F-actin specifically binds to the carboxy terminus of α-ENaC. A direct interaction between F-actin and the C-terminus of α-ENaC was further corroborated by F-actin co-sedimentation studies. This is the first study to report a direct and specific biochemical interaction between F-actin and ENaC.

INTRODUCTION

The amiloride-sensitive epithelial sodium channel (ENaC) is a member of the degenerin/epithelial sodium channel superfamily of ion channels. ENaC is expressed at the apical surface of polarized epithelia, and is in part responsible for maintaining proper salt and water homeostasis in the body. A great deal of information is known about ENaC’s biophysical properties once it is inserted into the apical surface of an epithelial cell’s plasma membrane. However, less is known about the proteins that interact with ENaC. Data from the literature indicate an interaction between ENaC and components of the apical membrane cytoskeleton. A partially purified ENaC complex from bovine renal epithelia copurifies with ankyrin, spectrin, and actin (1), suggesting these cytoskeletal proteins may be associated with ENaC. In addition, α-rENaC has been shown to bind to α-spectrin and this is mediated through direct interaction between α-spectrin’s SH3 domain and the second proline rich region in the C-terminus of α-rENaC (2). Electrophysiological data provide further support for an interaction between ENaC and the actin based cytoskeleton. In cell attached patches of A6 renal epithelial cells treated with the actin filament disrupter cytochalasin D, an induction of ENaC activity was observed (3), thereby suggesting that changes in the actin cytoskeleton affect the activity of ENaC. ENaC activation was also observed when short F-actin filaments were added to excised patches and this effect was increased with the addition of cytochalasin D and/or ATP. These effects were reversed by the addition of the G-actin binding protein, DNase I. In planar lipid bilayers, short F-actin filaments were demonstrated to increase the open probability of rENaC (4), whereas application of DNase I prevented the activation of rENaC. The application of gelsolin, a Ca²⁺-activated protein that severs actin filaments, and caps the plus end of the actin filament preventing
the repolymerization of actin and keeping it in a gel like state, was found to cause a sustained activation of rENaC. In addition, actin was required for the transient activation of rENaC by PKA and ATP when ENaC was reconstituted into planar lipid bilayers. These data indicate that a direct interaction between actin and ENaC may underlie the regulation of ENaC by short actin filaments. Identification of regions involved in a direct protein interaction between actin and α-ENaC has only been deduced from biophysical methods. Current evidence suggests that actin interacts with the C-terminal domain of α-ENaC.

A C-terminal truncation mutant (R613X) of α-rENaC was not responsive to the addition of actin. The single channel recordings of the α-rENaC residues 1-615 and α-bENaC residues 570-650, nullified the effect of actin normally seen with the chimeric α-bENaC (5). The deleted 14 amino acid sequence of α-bENaC has an 11 amino acid sequence identity to the same region of amino acids in α-rENaC’s C-terminus. This high degree of sequence identity suggests that this amino acid sequence in α-rENaC may also participate in the regulation of ENaC by actin. However, to date, there is no definitive biochemical evidence for a direct interaction between F-actin and α-ENaC.

In order to investigate the hypothesis that the actin cytoskeleton interacts directly with the carboxy terminus of α-ENaC, we have used a combination of gel overlay and F-actin co-sedimentation assays to demonstrate binding of actin to the C-terminus of α-ENaC. Actin was found to co-immunoprecipitate with α-ENaC from MDCK cell lysates, thereby providing in vivo data supporting an association between actin and ENaC. Moreover, co-localization of actin and α-ENaC in the apical membrane of MDCK cells stably expressing functional ENaC was demonstrated using laser scanning confocal microscopy. These three independent lines of evidence support an interaction between actin and α-ENaC which is mediated by the direct binding of actin to the carboxy terminus of α-ENaC.

MATERIALS AND METHODS

Cell culture - Stably transfected MDCK cells expressing the rat isoforms of αβγ-ENaC and MDCK parental cells were obtained as a kind gift from Drs. R. G. Morris and J.A. Schafer. The α-rENaC subunit was tagged with a single FLAG tag in the extracellular loop as described by Firsov et al. (7); both β and γ subunits were wild type. Cells were grown at 37°C in a 5% CO2 humidified incubator. Cells were initially grown in T75 flasks in Dulbecco’s Modified Eagle’s Medium (Life Technologies) supplemented with 10% FBS (HyClone), and antibiotics (penicillin and streptomycin 1%, G418 800 μg/ml, hygromycin 300 μg/ml, puromycin 5 μg/ml). Once the cells were at least 80% confluent, cells were seeded on poly-L-lysine coated semipermeable supports (Transwell 24 mm diameter, 0.4 μm pore size, Costar). Cells were used approximately 5-14 days later when polarized monolayers were formed. MDCK cells used for Western blots and co-immunoprecipitations were plated in 100 mm plastic Petri dishes. In some experiments, in order to increase ENaC expression, the media were supplemented with 2 μM dexamethasone (Sigma) and 2 mM sodium butyrate (Fluka) 24 h before the cells were harvested.

Electrophysiology - Whole-cell clamp bath solution was RPMI culture medium. The pipette solution was 100 mM K-gluconate, 30 mM KCl, 10 mM NaCl, 20 mM HEPES, 0.5 mM EGTA, free Ca2+ was less than 10 nM, 4 mM ATP, at a pH of 7.2. The bath contained serum-free RPMI-1640 cell culture medium (Na+ 133 mM, K+ 5.3 mM, Cl- 108.3 mM). These solutions approximate normal ionic gradients in situ. After formation of a giga ohm seal, the membrane within the seal was ruptured by an additional suction pulse. The whole-cell configuration was confirmed by an increase in the capacitance with no change in resistance. After the additional capacitance was balanced the cells were held at -60 mV and clamped to membrane potentials ranging from -160 mV to +40 mV sequentially for 1 second, returning to the holding potential (-60 mV) for 1 second between each test voltage. Currents were recorded digitally using pClamp hardware and software (Axon Instruments, Sunnyvale, CA). During bath perfusion to change
to amiloride-supplemented medium the cells were held at -60 mV and pulsed sequentially to -120 mV and +60 mV for 0.5 seconds, returning to the holding potential between each test pulse (see Figure 1C). This provided a continuous record and shows in real time the inhibition of inward current by amiloride. Single channel currents were recorded using the patch clamp technique. For cell-attached patches the pipette solution was RPMI culture medium, for outside-out patches the pipette solution was 150 mM KCl. In all cases the bath solution was RPMI culture medium. Data were recorded and analyzed using fetchex, fetchan, and pstat software (Axon Instruments, Sunnyvale CA).

**Immunocytochemistry -** MDCK cells were grown on poly-L-lysine coated semipermeable supports as described above. Culture media was aspirated from the monolayer, cells were rinsed twice with 1X phosphate buffered saline (PBS), [137 mM NaCl, 27.7 mM KCl, 1.5 mM KH2PO4, 8 mM Na2HPO4] and monolayers were then fixed with 3% paraformaldehyde (prepared from 20% EM Grade solution, Electron Microscopy Services) for 15 min at 37°C. Cells were rinsed three times with 1X PBS, and then permeabilized using 1X PBS + 0.1% Triton X-100 (PBST) for five minutes at room temperature. The blocking step was done with 1X PBS + 10% normal serum (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The wheat germ agglutinin/Alexa594 conjugate [WGA594] (Molecular Probes) was used at a concentration of 5 μg/ml diluted in 1X PBS and incubated with the samples for 10 min at room temperature. The anti-FLAG M2 antibody (Stratagene) was diluted 1:100 with 1X PBST from a stock of 2 mg/ml and incubated with the samples for 1 h at room temperature. Specificity of the anti-FLAG M2 antibody was demonstrated using the FLAG peptide (Sigma). The antibody was preabsorbed with the FLAG peptide, 10 μg peptide/1 μg of anti-FLAG M2 antibody for a minimum of 15 h prior to use. All phalloidin conjugates (Molecular Probes) were diluted 1:100 with 1X PBST from a stock concentration of ~6.6 μM, and incubated with the samples for 1 h at room temperature. Monolayers were then washed with 1X PBS, 3 times for 10 min at room temperature. Secondary antibody conjugated to Alexa488 or Alexa594 (Molecular Probes) was diluted 1:100 with 1X PBST from a stock of 2 mg/ml, and incubated with sample for 1 h at room temperature. After application of primary/probe and secondary antibody, samples were washed in 1X PBS, 3 times for 10 min each at room temperature. Counterstaining was performed using Hoechst 33258 (20 μg/ml in 1X PBS) for 3-5 min at room temperature. Cells were rinsed once with 1X PBS. Monolayers were mounted with 1% para-phenylenediamine in 1:9 (v/v) 1X PBS/glycerol and coverslipped. Images were viewed using laser scanning confocal microscopy (Leica DM IRBE microscope, mounted with a Leica TCS SP scanhead). The Leica DM IRBE microscope was equipped with oil, PlanApochromat 40x, 63x and 100x objectives. The 100x objective with a numerical aperture of 1.4 was used to acquire the final images. Visualization of blue fluorophores (Hoechst 33258) was achieved by using a dedicated UV laser (Coherent) for excitation at 350nm. Green fluorophore (Alexa488) excitation at 488nm was achieved by using an argon laser (Leica). Red fluorophore (Alexa 594) excitation at 568nm was achieved by using a krypton laser (Leica). Energy emission in the form of light by blue fluorophores (380nm-494nm), green fluorophores (500nm-575nm) and red fluorophores (596nm-722nm) was detected using three independent photomultiplier tubes. Color channels for the final double label images were captured sequentially and then merged using the Leica TCS NT software. Adobe Photoshop version 7.0 was used for image processing. All confocal microscopy was done at the University of Alabama’s High Resolution Imaging Facility and the V.A. Hospital, Birmingham, Alabama.

**SDS-Polyacrylamide Gel Electrophoresis, Immunoprecipitations/Co-immunoprecipitations, Immunoblotting -** Whole cell lysates prepared from MDCK cells stably expressing ENaC and MDCK parental cells were used. Cells were grown in three 100 mm plastic Petri dishes until confluent. Cells were washed twice with 2 ml of cold, 1X PBS. Petri dishes were placed on ice for 10 min with 1 ml each of 1X lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl and 1% TritonX-100) supplemented with Complete Protease Inhibitor cocktail (Roche Molecular Biochemicals). Cells were scraped from the Petri dishes and placed into
microcentrifuge tubes on ice. Lysates were sheared a minimum of three times with a 22 gauge needle, and incubated on ice for 1 h. Sheared lysates were then spun at 15,800 x g at 4°C for 5 minutes. The supernatant was removed and a BCA protein assay (Pierce) was performed to quantify the amount of total protein in the samples. A maximum of 200 µg of whole cell lysate diluted in 1X PBS was used per immunoprecipitation reaction and incubated overnight at 4°C on a rotator with 3 µg of anti-FLAG mAb (Stratagene), 40 µl of a 50% slurry of protein G agarose beads (Roche Molecular Biochemicals) and Complete Protease Inhibitor cocktail (final volume of 500 µl). After overnight incubation, beads were centrifuged for 2 min at 15,800 x g. The supernatant was aspirated, and beads were washed and pelleted three times (2 min at 15,800 x g) in 1X lysis buffer supplemented with Complete Protease Inhibitor cocktail. Samples were diluted 1:1 (v/v) with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, 5% β-mercaptoethanol), heated at 95°C for 5 minutes, and separated by SDS-PAGE with constant voltage at room temperature. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The Western transfer was done in running buffer (25 mM Tris base pH 8.0, 192 mM glycine, 1% (w/v) SDS, 20% (v/v) methanol) at 4°C for 1 h at constant voltage. Blots were either blocked in 1X TBST (10 mM Tris base, pH 8.0, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk overnight at 4°C or for 1 h at room temperature. Following immunoprecipitation with the mAb anti-FLAG antibody, Western blot detection of alpha rENaC was performed using anti-FLAG mAb (1:500 dilution) or a rabbit polyclonal anti-alpha ENaC antibody (2 µg/ml final concentration) (Affinity BioReagents). Specificity of the anti-alpha ENaC antibody was demonstrated using its immunizing peptide (Affinity BioReagents). The antibody was preabsorbed with its immunizing peptide 2 µg peptide/1 µg of alpha ENaC antibody for a minimum of 15 h prior to use. Primary antibodies were diluted with 1X TBST/1% non-fat dry milk and incubated with blots for 1 h at room temperature. Blots were washed a minimum of 3x with 1X TBST, 10 min each at room temperature. Secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories) were used at dilutions of 1:10,000 or 1:20,000 in 1X TBST and incubated for 1 h at room temperature. Blots were washed a minimum of 3x with 1X TBST, 10 min each at room temperature. Immunoreactivity was visualized using enhanced chemiluminescence (Super Signal West Pico, Pierce) and imaged onto Kodak X-OMAT AR film (Fisher Scientific). Controls for immunoprecipitations consisted of using an equal concentration of ChromPure normal IgG serum (Jackson ImmunoResearch Laboratories) as there was primary antibody. Co-immunoprecipitation of α-rENaC with actin was performed as described above with the following exceptions: 1) the lysis buffer used had the following composition: 50 mM Tris base, 15 mM Na 2HPO 4, 150 mM NaCl, 0.5% TritonX-100, 0.5% Deoxycholate, pH 7.4. 2) following the BCA assay, the 200 µg of supernatant was precleared with 50 µl of protein A agarose beads and 50 µl protein G agarose beads for 2 h at 4°C. 3) After preclearing, the samples were spun, and the anti-FLAG antibody alone was added for overnight incubation at 4°C. 4) The α-rENaC/FLAG complex was captured by the addition of 40 µl of protein G agarose and incubated for 2 h at 4°C. 5) The beads were washed 3x with the lysis buffer + 500 mM KCl, pH 9.1. 6) The co-immunoprecipitation control consisted of an anti-His antibody (Santa Cruz) added in equivalent concentration as the primary anti-FLAG antibody. The monoclonal anti-actin antibody (Chemicon) was used at a 1:1,500 dilution for 1 h at room temperature. Immunoblotting and primary antibody detection for the co-immunoprecipitations was done exactly as described above.

Construction of GST ENaC fusion constructs - PCR amplification of the γ-rENaC C-terminus (aa A563-I650, bp 1,785-2,051), the α-rENaC N-terminus (aa M1-N90, bp 82-351), and the α-rENaC C-terminus (aa R613-L698, bp 1,918-2,178) consisted of using full length α and γ-rENaC cDNAs (a kind gift of Dr. B. Rossier, Lausanne, Switzerland). The PCR reaction (50 µl total volume) consisted of: 5 µl of 10X buffer, 0.8 µl of Mg 2+ (1.5 mM), 1 µl of dNTP (25 mM per nucleotide), 1 µl of primer (15 pmoles per primer), 100 ng of DNA template, and 0.5 µl of native Pfu
polymerase (Stratagene). PCR products were analyzed on 2% agarose gels (NuSeive 3:1 agarose) for gel purification (Qiagen). After purification, the insert was subcloned into the pCR4.0 TOPO-TA vector (Invitrogen), and transformed into chemically competent TOP10 *Escherichia coli* (*E. coli*) (Invitrogen). Transformed bacteria were grown overnight at 37°C on LB/ampicillin plates. Positive clones were screened by restriction enzyme digestion, grown up overnight again, and the plasmid DNA was isolated (Promega). Following confirmation by sequences, the amplified cDNA was excised from the pCR4.0 vector and ligated into linearized pGEX 5X-1 GST expression vector (AP Biotech). The ligation products were then transformed into the JM109 maintenance strain (Promega), plated overnight and screened for positive clones by restriction enzyme digestion. DNA from positive clones was isolated (Qiagen), sequenced and then transformed into *E. coli* BL21-CodonPlus-RP (Stratagene) cells for the production of GST fusion proteins.

**Generation and Purification of GST fusion proteins** - GST α-hENaC C-terminal fusion protein (a generous gift of Dr. F.J. McDonald, Dunedin, New Zealand), GST γ-rENaC C-terminal, GST α-rENaC N terminal, and GST were produced in *E. coli* BL21-CodonPlus-RP (Stratagene). Cells were induced with 0.1mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. Bacterial cultures were spun at 10,000 x g for 10 min at 4°C. Bacterial pellets were snap frozen with liquid N2 and stored overnight at -80°C. Pellets were lysed with BugBuster (Novagen) in the presence of benzonase (Novagen) and Complete Protease Inhibitor cocktail (Roche Molecular Biochemicals) for 20 min at room temperature or at 4°C for 45 min, then spun at 16,000 x g for 20 min at 4°C. Supernatant was poured over a glutathione-Sepharose 4B (AP Biotech) gravity flow column five times. Bound GST fusion protein or GST was eluted with 10 mM reduced glutathione (Sigma Chemical) in three, 2 ml fractions. The purified fractions were assayed by SDS-PAGE and the gel was stained with Gel Code (Pierce). The fractions were then pooled, concentrated, and dialyzed overnight at 4°C in 1X PBS with three buffer changes.

**Labeling and Polymerization of Non-muscle actin** - Non-muscle actin (Cytoskeleton) was labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester (Molecular Probes) according to the manufacturer. The polymerization of the non-muscle actin was done as previously described by Hitt et al. (8)

**Gel Overlays** - Samples were heated at 95°C for 5 minutes and separated using 12% Tris/glycine gels with constant voltage at room temperature. Proteins were transferred onto PVDF membrane in running buffer (25 mM Tris base pH 8.0, 192 mM glycine, 20% (v/v) methanol) at 4°C for 1 h at constant voltage. Gel overlays were blocked in 1X TBST/ 5% nonfat dry milk overnight at 4°C. The blots were probed with 25 μg/ml of F-actin/Alexa488 in 1X TBST/ 5% nonfat dry milk for 2 h at room temperature, washed briefly in 1X TBST, allowed to air dry, and scanned using a FujiFilm FLA-5100 Scanner (FujiFilm). Images were processed using Adobe Photoshop 7.0

**Non- Muscle F-Actin Co-sedimentation** – Non-muscle F-actin co-sedimentation experiments were done according to the manufacturer’s instructions (Cytoskeleton). All samples were incubated at room temperature for 30 min. Briefly, 40 μl of a 23 μM non-muscle F-actin stock was incubated with 10 μl of GST α-hENaC C-terminal fusion protein (0.4 μg final concentration). Negative controls consisted of 40 μl of F-actin buffer incubated with 10 μl of GST α-hENaC C-terminal fusion protein (0.4 μg final concentration), and an equal amount of GST protein that was used for the GST α-hENaC C-terminal fusion protein sample. Positive controls consisted of 1 μl of α-actinin (2.5 μg), 40 μl of non-muscle F-actin, 8 μl of F-actin buffer, and 1 μl of Tris-HCl. Following the 30 min incubation, samples were subjected to ultracentrifugation for 1.5 h, 150,000 x g at 24°C. Supernatant was removed, and pellets were resuspended in 50 μl of Laemmli sample buffer, and heated at 95°C for 5 minutes. Equal volumes of supernatant and pellet were loaded into the wells, separated using 12 % Tris/glycine gels with constant voltage at room temperature. Gels were stained using GelCode (Pierce).
RESULTS

Determination of Functional ENaC – Wild type MDCK cells do not endogenously express amiloride-sensitive epithelial sodium channel (9;10). MDCK cells stably transfected with rat α,β,γ-ENaC were examined by whole-cell patch clamp. Three stably transfected MDCK cells (induced to increase expression of ENaC) were whole-cell clamped. Each cell had inward sodium current that was inhibited by 2 µM amiloride, thereby confirming the presence of ENaC in the plasma membrane. Figure 1 (A,B) shows a representative example of whole-cell currents before and after treatment with amiloride. Figure 1C shows the amiloride block in a real time pulse record. Figure 1D is the summary of the amiloride-blockable current-voltage data. The amiloride-sensitive current reversed at a positive membrane potential, indicating sodium selectivity. Figure 1B shows the slight reduction in outward current after amiloride treatment. This may be due to inhibition of outward potassium flux through ENaC. The result is a shift to the left in the I-V relation.

In the cell-attached configuration, small single channel currents with relatively long open times were observed (Figure 1E). Moreover, cAMP characteristically increased channel activity (NPo 0.5 vs. 1.5). The single-channel conductance was 8 pS, which is typical of human ENaC channels. (11) To confirm independently the identity of the single channels, outside-out patches were formed, and amiloride (100 nM) was added to the bath solution (Figure 1F). 100 nM is slightly above the IC50 (75 nM) for inhibition of ENaC. This concentration was chosen because it would produce a partial inhibition of the channels, changing the long open times into rapidly transitioning channels due to the binding and unbinding of the inhibitor. Based on these observations (conductance and appropriate inhibition by amiloride) we conclude that the stably transfected MDCK cells expressed functional ENaC in their apical plasma membrane.

Co-localization of alpha ENaC with the cortical F-actin cytoskeleton - Initially, stably transfected MDCK cells expressing the alpha rENaC subunit with a FLAG epitope tag on the extracellular loop, and wild type beta and gamma rENaC subunits, were labeled with a monoclonal anti-FLAG epitope antibody to determine the membrane localization of α-ENaC. Figures 2A-E show representative images of the pattern of expression for α-rENaC in this cell line. As these images illustrate, the level of α-rENaC expression varied between cells. Figure 2E is a XZ reconstruction, illustrating that ENaC immunoreactivity was primarily situated in the apical domain of this cell line. In contrast to the anti-FLAG antibody, the peptide competition controls (Fig. 2 F,G) exhibited little or no detectable immunoreactivity at the surface of the monolayer (Fig. 2F) or within the cytoplasm (Fig. 2G). In order to determine where the apical anti-FLAG immunoreactivity was located relative to the apical plasma membrane, double labeling experiments were performed. Figure 2H is a XZ reconstruction of a monolayer labeled with anti-FLAG antibody to detect α-rENaC. The monolayer was labeled with wheat germ agglutinin (WGA) conjugated to Alexa594 (WGA594) prior to fixation to delineate the apical plasma membrane (Fig. 2I). Figure 2J is an overlay of Panels H and I, which demonstrates co-localization of α-rENaC with WGA within the apical plasma membrane.

To localize the cortical F-actin cytoskeleton with respect to the apical membrane in MDCK cells stably expressing ENaC, monolayers were labeled with WGA594 and the specific F-actin probe, phallolidin conjugated to Alexa488. Figures 3A-D are optical sections from a Z-stack of MDCK ENaC cells. As the optical sections were taken deeper into the cells, the fluorescence from the WGA594 largely disappeared, leaving only the basolateral labeling of the phallolidin/Alexa488 in the majority of the field of view. An XZ reconstruction (Fig. 3E-G) revealed co-localization of actin and WGA in some of the cells, demonstrating that a population of F-actin is associated with the apical membrane. We then performed double labeling experiments to determine if α-rENaC and F-actin are co-localized in this model system. The representative XZ reconstructions in Figs. 4C and 4F, shows the overlay of the FLAG (Fig. 4A & 4D) and phallloidin (Fig. 4B & 4E) signals. When the two signals are merged, partial co-localization can be observed, indicating that both F-actin and
α-rENaC segregate to the same region of the apical domain.

Western blotting of alpha ENaC - In order to examine if α-rENaC and actin are found in the same protein complex, we attempted to co-immunoprecipitate FLAG tagged α-ENaC and actin from the MDCK cell line. To first demonstrate that we were able to immunoprecipitate specifically FLAG tagged α-ENaC, we used the monoclonal anti-FLAG antibody; the alpha subunit was immunoprecipitated using the anti-FLAG antibody and the resulting immunoblot was then probed with the same primary antibody (Fig. 5A). As shown in lane 3, FLAG tagged α-ENaC was only detected by the anti-FLAG antibody in lysates immunoprecipitated with the anti-FLAG antibody. It was not detected in the beads only control (Lane 1), the non immune mouse IgG control (Lane 2), or when blots were probed with the secondary antibody alone (Lanes 4-6). To further demonstrate that the 100 kDa polypeptide immunoprecipitated by the anti-FLAG antibody was indeed alpha rENaC, samples were immunoprecipitated with the anti-FLAG antibody and the blot was probed using a commercially available anti-α-rENaC antibody (Figure 5B). In addition to the stably transfected MDCK ENaC cell lysate (designated E), lysates prepared from the original MDCK parental strain (designated P) were subjected to immunoprecipitation with the anti-FLAG antibody. The parental strain has previously been reported not to express ENaC as assayed by RT-PCR (12). As shown in lane 1, of Fig. 5B, a band at ~100 kDa was observed, whereas no signal was detected in lane 2 from the parental cell line. In order to demonstrate the specificity of this antibody, a peptide competition experiment was performed using replicates in lanes 3 and 4. The samples were immunoprecipitated with the anti-FLAG antibody, and the blot was probed with the primary antibody which had been preabsorbed with its immunizing peptide. Upon development of the blot, the ~100 kDa band previously observed in lane 1 from the ENaC expressing MDCK cell line was not detected.

Association of alpha ENaC with F-actin in vivo - In order to determine if F-actin is found in the same protein complex as the alpha subunit within the MDCK cell line stably expressing FLAG tagged ENaC, we attempted to co-immunoprecipitate actin with FLAG tagged α-ENaC. Cell lysates prepared from MDCK cell line stably expressing FLAG tagged α-ENaC were immunoprecipitated with anti-FLAG antibody, an irrelevant mouse monoclonal antibody, or beads alone. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane and blots were probed with a monoclonal anti-actin antibody. As can be seen in Figure 6, no actin signal was detected in the beads alone control (Lane 1). The anti-actin antibody detected a strong signal at about 45kDa (Lane 3) corresponding to the molecular mass of actin, in the cell lysate immunoprecipitated with the anti-FLAG antibody. A faint signal was observed when an irrelevant mouse monoclonal antibody (mAb anti-His) was substituted for the anti-FLAG antibody in the immunoprecipitation. We anticipated we would observe a small amount of “background” in lane 2 in the anti-His control lane (Lane 2), as actin interacts with IgG (13). This report demonstrated that the so called “non-specific background” often seen in this type of experiment is actually an interaction between actin and IgG. Furthermore, the authors of the study showed the amount of IgG that would coprecipitate with actin would decrease as the pH of the buffer was increased (13). Therefore, in order to minimize this interaction in our experiments, the beads were washed two times with a high salt buffer with a pH of 9.1. An additional control experiment done exactly as the co-immunoprecipitation experiments described above, but using MDCK parental cell lysates, revealed no detectable actin following treatment with anti-FLAG antibody (unpublished data). This observation suggests the actin signal seen in the anti-FLAG lane is due to the co-immunoprecipitation of actin with α-rENaC, rather than an interaction between actin and IgG. These data demonstrate that F-actin and α-rENaC are found within the same protein complex.

The carboxy terminus of alpha hENaC binds directly to non-muscle F-actin in gel overlays - Electrophysiological studies have suggested that F-actin interacts with the C-terminus of the alpha subunit of ENaC (5;6). However biochemical data demonstrating a direct
interaction between ENaC and actin are lacking. To determine if F-actin binds to α-ENaC’s C-terminus, we performed gel overlay assays, a widely used biochemical technique to examine if actin binds directly to another protein.

A representative sample of the eluted GST α-hENaC C-terminal fusion protein (1.0 µg of full length fusion) stained with Gel Code is shown in Fig. 7A. In Fig. 7A, the arrow points to the full length fusion protein, and the asterisk marks degradation products from the fusion protein. As shown in Figure 7B, full length GST α-hENaC C-terminal (Lane 1) or GST γ-rENaC C-terminal (Lane 5) fusion proteins, which were used as additional negative controls. Whole cell MDCK ENaC lysates, which contain numerous actin binding proteins, were used as positive controls for actin binding (Lane 4). Fig. 7B (Lanes 1,2,3, and 5) and 7C, binding of non-muscle actin/Alexa488 to a protein below the 75kDa molecular mass marker was observed. The identity of this protein was not determined, but is believed to be a bacterial protein that is commonly co-purified with the GST fusion proteins (14). Fig. 7C shows a representative microtubule and non-muscle actin/Alexa488 competition assay, which was used to determine if the binding of actin is specific or nonspecific for the C-terminus. Actin, like microtubules, is a negatively charged biopolymer. If the binding is due to nonspecific interactions, then the microtubules will bind to the fusion protein and no signal would be observed, whereas if the binding interaction is specific, then a signal from the non-muscle actin/Alexa488 would still be observed. As shown in Fig. 7C, non-muscle actin/Alexa488 did bind to the GST α-hENaC C-terminal fusion protein in the presence of microtubules. A second competition control, Fig. 7D was conducted to establish that binding was not a result of the Alexa488 and its linker group. Gel overlay membrane strips were incubated with non-muscle F-actin/Alexa488 (25 µg/ml) in the presence of two different amounts of unlabeled non-muscle F-actin (Lane 1, 100 µg/ml and Lane 2, 1 mg/ml) The binding of the F-actin labeled with Alexa488 to the GST α-hENaC C-terminal fusion protein was observed to decrease with an increased amount of unlabeled actin as shown in Figure 7. As shown in Figure 7E, full length GST α-rENaC C-terminal (1.0 µg of full length fusion denoted by the arrow, an asterisk denotes a degradation product) was observed to bind non-muscle F-actin in gel overlays as well.

The carboxy terminus of alpha hENaC co-sediments with non-muscle F-actin in actin co-sedimentation assays - Actin co-sedimentation assays were used to corroborate the results of the actin overlay assays. In order to investigate if α-ENaC’s C-terminus directly binds to F-actin we used a human α-ENaC fusion protein. The F-actin binding experiments were carried out with the human α-ENaC fusion protein because the full length rat α-ENaC fusion protein undergoes a severe amount of degradation. This results in a very small yield of full length GST α-rENaC C-terminal fusion protein. The 14 amino acid putative actin binding domain has a 78% sequence identity between the rat and human isoforms of α-rENaC. Only 3 amino acids in the putative actin binding domain are different between the human and rat ENaC isoforms. All three amino acids are conserved substitutions; the conserved substitutions do not change the charge in this region. Actin co-sedimentation assays were performed to demonstrate that the binding between the GST α-hENaC C-terminal fusion protein and non-muscle F-actin was not due to the linear conformation that the fusion protein adopted when run through the SDS-PAGE gels used in the gel overlay experiments. The experiment illustrated in Figure 8A is a control sedimentation of the GST α-hENaC C-terminal fusion protein in the absence of actin to determine in which fraction the fusion protein comes down following sedimentation. The majority of the fusion protein was detected in the soluble fraction (S), and to a lesser extent some fusion protein was detected in the pellet (P). The arrow denotes the full length GST α-hENaC C-terminal fusion with a mass of approximately 35kDa. An asterisk denotes degradation products of the full length length GST α-hENaC C-terminal fusion protein. The protein detected shortly below the 75kDa molecular mass marker, which is detected in all the soluble fractions, was
unidentified. However, when a GST fusion protein is eluted from a column during purification, often an assortment of bacterial proteins can co-purify with the GST fusion protein (14). DnaK is a bacterial protein with a mass of ~70kDa and is often observed when GST fusion proteins are eluted from columns (14). As shown in Figure 8B, the GST α-hENaC C-terminal fusion protein but not GST alone was found to co-sediment with non-muscle F-actin. A small amount of GST α-hENaC C-terminal fusion protein was observed in the P lane of the GST sample. This was a result of a very small amount of spill over from sample loading of an adjacent S lane of GST α-hENaC C-terminal fusion protein. F-actin was detected as the large bands observed in the P lanes with a mass of approximately 45kDa. The positive control, α-actinin, a known actin binding protein with a mass of approximately 100kDa, was observed to co-sediment with non-muscle F-actin. These data demonstrate that F-actin binds to the GST α-hENaC C-terminal fusion protein in vitro.

**DISCUSSION**

The mammalian cytoskeleton is comprised of three major protein families: microfilaments, microtubules, and intermediate filaments, as well as numerous cytoskeletal associated proteins (15). Together they contribute towards a cell’s shape, motility, signal transduction, vesicular trafficking, and ion channel/transporter/receptor function. The actin cytoskeleton has been shown to interact both directly and indirectly with ion channels and membrane transport proteins. In addition to functioning as an anchor for ion channels/transporters within the plasma membrane (16), the actin cytoskeleton participates in the vesicular trafficking of ion channels and transporter proteins to the membrane (17;18). To date, the majority of the literature has described indirect protein interactions between the actin cytoskeleton and ion channels and transporters. These protein interactions are often mediated through a protein linking actin to the integral membrane protein. For example, CFTR is linked to the cortical actin cytoskeleton through a protein scaffolding complex composed of EBP-50 and ezrin (19). However, there is increasing evidence that the actin based cytoskeleton also directly interacts with a number of ion channels, transporters, and receptors such as (20-23).

Ion channels and transporters perform a variety of functions, from action potential propagation in the nervous system, to allowing the passage of ions across a cell’s membranes to maintain extracellular fluid homeostasis. However, if and how, the actin cytoskeleton influences the function of an ion channel or transporter by either indirect or direct binding to the integral membrane protein is poorly understood.

Although there are several reports regarding the biophysical affects of actin on ENaC (3-6;24-26), there are no reports in the literature that have attempted to elucidate biochemically the nature of the ENaC/actin relationship. Previous biophysical studies offer insight into the nature of the ENaC/actin interaction. The C-terminus of α-ENaC has been shown to contribute to the modulation of the channel by actin (5). Through the use of chimeric constructs composed of α-rENaC and α-bENaC along with site directed mutagenesis, a conserved 14 amino acid region in the C-terminus of α-bENaC has been shown to contribute specifically to actin’s modulation of ENaC (6). In the present study, we have investigated the question of whether or not actin binds to the C-terminus of α-ENaC.

ENaC is expressed in the apical domain of polarized epithelia. However, because ENaC is found in such small amounts in native epithelia (27), we used a MDCK cell line stably transfected with wild type, rat isoforms of alpha with a FLAG tag on its extracellular domain, and beta and gamma ENaC for our studies. Figure 1 demonstrates the ENaC expressed in this MDCK cell line forms functional amiloride-sensitive sodium channels at the apical surface of the plasma membrane domain, and that they resembled native α,β,γ ENaC channels in their biophysical properties. The conductance was 8 pS at a transmembrane potential of 60 mV, NPo was increased by cAMP, and the channels exhibited the appropriate inhibition at the appropriate concentration by amiloride.

This model system allowed us to examine if functional ENaC at, or near, the apical domain of the MDCK monolayers co-segregated with the
cortical F-actin cytoskeleton. Initial single label experiments were performed to examine the normal pattern of expression and localization of the FLAG tagged α-ENaC subunit. The pattern of α-ENaC expression within this cell line is heterogeneous, with some cells having higher expression levels than others. However the localization of α-ENaC remained consistent through all samples, as seen in Fig. 2E, where the immunofluorescence is apical and subapical. We also performed double label experiments to determine if the apparent apical labeling of α-ENaC was in fact in the apical plasma membrane, or if the labeling was completely below the cell surface. We observed α-ENaC in the apical membrane as well as below the apical domain, as shown in Figure 2J. The localization of the alpha subunit, found both in the apical plasma membrane and subapically, is consistent with previous studies utilizing other monolayer forming epithelia stably transfected with ENaC (12;28). In addition, the α-ENaC localization we observed is also consistent with observations reported in native epithelia from the cortical collecting duct of the kidney (29-32). Immunoprecipitations for α-ENaC (Fig. 5 Panels A & B) were carried out to confirm the single label experiments. We did a second series of double label experiments (Fig. 3 Panels A-G) to examine the position of the F-actin immunofluorescence signal relative the apical plasma membrane itself. As expected, the cortical F-actin cytoskeleton was observed just beneath the apical plasma membrane in our model system. Figure 4A-F are two different double label experiments that were performed to see if α-ENaC colocalized with the cortical F-actin cytoskeleton. Fig. 4C and Fig. 4D both demonstrate that partial co-localization can be observed, indicating that both F-actin and α-ENaC segregate to the same region of the apical domain. This is a significant observation because this places these two proteins in the same place, suggesting the possibility of an interaction between α-ENaC and the cortical actin cytoskeleton. While the immunocytochemistry was encouraging, we needed to see if α-ENaC and F-actin exist as a complex, and in fact they do. We used whole cell lysates in these experiments for immunoprecipitating α-ENaC. Figure 6 shows an actin-like band at approximately 45kDa. The α-ENaC that was immunoprecipitated was in all likelihood a mixture of both α-ENaC inserted into the apical membrane, and α-ENaC residing in intracellular pools, this datum supports the subapical co-localization of F-actin and α-ENaC observed in Fig. 4F. These data demonstrate that α-ENaC is located in the apical domain with the cortical actin cytoskeleton, and the co-immunoprecipitation of α-ENaC and actin from MDCK cell lysates corroborates an in vivo interaction between these two proteins.

The exact functional role(s) of interaction between the cortical actin cytoskeleton and ENaC is unresolved. However, there are some possibilities from other studies that may apply here. Actin has been shown to function structurally as an anchor for ion channels/transporters (16;33), and to participate in the vesicular trafficking of ion channels/transporters (17;18) in response to hormonal stimulation. This may be a possibility for ENaC as well, as studies have shown ENaC trafficking in response to hormonal stimulation (32;34-36). Hormonal-mediated ENaC trafficking may involve the actin cytoskeleton. Another line of thought is that the cortical actin cytoskeleton functions as a physical barrier to vesicular exocytosis (37-40). In doing so, it remains in a polymerized state and prevents vesicles containing ion channels/transporters from coming in contact with the apical membrane, and thus preventing vesicular fusion and channel insertion into the surface of a cell. Alternatively, vesicles containing ENaC could be trafficked or held in place by the cortical actin cytoskeleton. In toad urinary bladder, the cortical actin cytoskeleton forms a meshwork of short filaments just under the apical surface when observed with an electron microscope (41), this is probably true for cortical collecting duct cells in the mammalian kidney. This would in turn lend support to previously reported observations which demonstrated that short actin filaments had an effect on ENaC by increasing the channel’s open probability and decreasing its conductance (4;6). It may be that short actin filaments located subapically directly bind to ENaC in response to hormonal influences. In toad urinary bladder antidiuretic hormone (ADH) causes an increase in the water permeability of epithelia in both mammals and anurans (42). The ADH also causes a
reorganization of F-actin which is believed to allow vesicular fusion to occur. A similar mechanism may occur in ENaC-containing epithelia. In addition, GLUT4 translocation has been reported to occur via an insulin-stimulated remodeling of the cortical F-actin cytoskeleton (18), and insulin-stimulated GLUT4 translocation requires PI-3 kinase function as well (18). Blazer-Yost et al. have observed in renal cells that insulin-stimulated trafficking of ENaC required PI-3 kinase (34). The authors of that study did not report on any aspects of the cytoskeleton; a possible mechanism of ENaC translocation may in part involve the remodeling of the cortical F-actin cytoskeleton in response to hormonal stimuli.

In order to answer the question, does α-ENaC’s C-terminus bind directly to F-actin, we carried out gel overlays and F-actin cosedimentation assays using a GST α-hENaC C-terminal fusion. The 14 amino acid putative actin binding domain contained within the C-terminus of alpha has a 78% sequence identity between the rat and human isoforms, with only 3 conserved amino acid substitutions that are different in this region. Figure 7B, Lane 3 shows a strong signal at approximately 35kDa where F-actin/Alexa488 is bound to the full length GST α-hENaC C-terminal fusion protein. A weaker signal was observed below the full length fusion protein where the F-actin/Alexa488 was bound to degradation products of the fusion protein. In Fig. 7E we also observed binding of F-actin to a full length GST α-rENaC C-terminal fusion protein. The full length fusion proteins are denoted by the arrowheads. In Fig. 8, in the negative control (no actin) the GST α-hENaC C-terminal fusion is almost entirely in the soluble fraction, with a small amount in the pellet. When this fusion is incubated and spun down with F-actin, approximately half of the fusion now appears in the pellet with F-actin, and the negative GST control is found almost entirely in the soluble fraction. One explanation for this observation could be that not all of the GST α-hENaC fusion protein made is in the proper conformation, thus preventing it from binding to the F-actin. These binding studies demonstrate that F-actin binds directly and specifically to the C-terminus of α-ENaC.

Our results support the biophysical data that suggest a functional interaction between α-ENaC’s C-terminus and F-actin. We demonstrate for the first time a direct and specific interaction between F-actin and ENaC.

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FOOTNOTES

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The abbreviations used are: ENaC, epithelial sodium channel; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Triton X-100; WGA, wheat germ
agglutinin; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline Tween-20; GST, Glutathione S-Transferase; CFTR, cystic fibrosis transmembrane conductance regulator; EBP-50, ezrin-radixin-moesin binding phosphoprotein 50; ADH, antidiuretic hormone; GLUT4, glucose transporter-4, Ms IgG; mouse immunoglobulin.

FIGURE LEGENDS

Fig. 1. Determination of Functional ENaC in stably transfected MDCK ENaC cell line. Panel A) Basal whole-cell clamp record from an MDCK cell induced to express ENaC. Panel B) Whole-cell clamp record from the same cells as shown in A, after exposure to 2 µM amiloride. Panel C) Real time whole-cell clamp record showing the immediate and specific (for inward current) inhibition produced by 2µM amiloride. Panel D) Current-voltage relation of the difference in current before and after treatment with amiloride. These measurements were made by digitally subtracting (point for point) the currents recorded before and after amiloride treatment. Panel E) Single channel recordings from two cell-attached patches on MDCK cells stably transfected with rat αβγ ENaC. The pipette solution was RPMI culture medium, thereby reflecting the normal ionic gradient across the patch for sodium. Unstimulated the Basal: Npo = 0.5: where N = 2, and Po = 0.25. After stimulation with 40 µM 8-CPT-cAMP, the Npo increased to 1.5, where N remained at 2, and Po increased to 0.75. Panel F) Single channel record shown partial inhibition of the channel activity by a low concentration of amiloride. The rapid closed to open transitions were induced by the binding and unbinding of the inhibitor. Whole-cell data is representative of 3 experiments, single channel data is representative of 1 experiment.

Fig. 2. Localization of α-rENaC in MDCK ENaC cell line and co-localization of α-rENaC with the apical plasma membrane
Confocal micrographs from a Z-stack of MDCK ENaC cells probed for FLAG tagged α-rENaC using anti-FLAG antibody. Panels A-D show the pattern of immunoreactivity for FLAG tagged α-rENaC at different optical sections. Panel A 0.97 µm into the Z stack, Panel B 1.95 µm, Panel C 3.84 µm and Panel D 10.34 µm. Immunoreactivity is brightest at the surface of the monolayer (Panel A), and begins to decrease significantly at the level of the nuclei (Panel D). Panel E is an XZ reconstruction taken from the region demarcated by the white line in Panel A. Immunoreactivity for α-rENaC is localized primarily in the apical domain. Panels F & G are FLAG peptide competition control images. Panel F shows the signal due to the FLAG antibody was competed away when the FLAG antibody was preincubated with the FLAG peptide, demonstrating the specificity of this antibody. The XZ reconstruction in Panel G has no detectable immunoreactivity in the apical domain when compared to Panel E. Panels H-J are confocal micrographs of an XZ reconstruction of a monolayer dual labeled for FLAG epitope tagged α-rENaC (anti-FLAG antibody) (Panel H), apical plasma membrane (WGA/Alexa594) (Panel I) and merged in Panel J. Panel J shows co-localization of α-rENaC with the apical plasma membrane, revealing that α-rENaC is expressed in the apical plasma membrane of the MDCK ENaC cells. Nuclei were stained with Hoechst 33258. All settings for the lasers, Leica TCS SP scanhead and Leica TCS NT software were the same when acquiring the images for the control and experimental samples. Scale bar 10 µm. The single label experiment is representative of work repeated 5 times, and the double label experiment is representative of work repeated 3 times.

Fig. 3. Localization of F-actin with respect to the apical plasma membrane in MDCK ENaC cells. Confocal micrographs from a Z-stack of MDCK ENaC cells labeled with Wheat Germ Agglutinin/Alexa594 which labeled the apical plasma membrane, and phalloidin/Alexa488 which binds specifically to F-actin. Panels A-D show the localization of the cortical F-actin cytoskeleton with respect to the apical plasma membrane. Panel A is at the surface of the monolayer with respect to
WGA/Alexa594 labeled cells in the center. Panel B is 0.48 μm deep, Panel C 0.97 μm and Panel D 1.46 μm. The Z-stack was sectioned in 0.4884 μm steps. Panels E-G represent a single XZ reconstruction from the same cells. The white line across Panel A represents the region that Panels E-G were reconstructed from. Nuclei were stained with Hoechst 33258. Scale bar 10 μm. This experiment is representative of work repeated 3 times.

**Fig. 4. Co-localization of F-actin and α-rENaC in stably transfected MDCK cells.**

Panels A,D) Immunoreactivity of FLAG α-rENaC showing apical as well as some subapical labeling. Panels B,E) Labeling with phalloidin/Alexa 594 to visualize F-actin. Panels C,F) Merged images of Panels A & B, and Panels D & E respectively showing that F-actin and α-rENaC co-segregate to the same region of the apical domain in vivo. Scale bar 10 μm. Samples were fixed with 3% paraformaldehyde at 37°C, Triton X-100 permeabilized and blocked with 10% normal goat serum. This experiment is representative of work repeated 4 times.

**Fig. 5. Immunoprecipitation of α-rENaC from MDCK rENaC whole cell lysates.**

Panel A) Lane 1 Protein G agarose beads and MDCK ENaC lysate. Lane 2 immunoprecipitated with mouse IgG (3 μg). Lane 3 immunoprecipitated with mouse monoclonal anti-FLAG (3 μg). This lane has a band at approximately 100kDa which corresponds to alpha rENaC. The Western blot with lanes 1-3 was probed with mouse mAb anti-FLAG (1:500). Lanes 4, 5 and 6 were immunoprecipitated as described for lanes 1, 2 and 3 respectively, however, the blot was probed only with the polyclonal goat anti-mouse-HRP conjugate. Notice in lane 6, the 100kDa signal is not detected when the blot is probed with the secondary antibody alone. Samples were immunoprecipitated overnight at 4°C on a rotator with Protein G agarose beads. Samples were separated on 8% SDS-PAGE gel, transferred to PVDF, blocked with 1X TBST+5% non fat dry milk. Immunoblots were probed with mouse mAb anti-FLAG primary antibody, or the primary antibody was omitted. The secondary antibody used was a polyclonal goat anti-mouse-HRP conjugate. The signal was visualized using enhanced chemiluminescence. Panel B)

Detection of α-rENaC using an alternative antibody. Lane 1, α-rENaC was immunoprecipitated from MDCK ENaC lysates (E) exactly as described in Panel A. Lane 2, MDCK Parental lysates (P) were used as a control for antibody specificity. Lanes 1 and 2 were probed with a rabbit polyclonal α-ENaC (2 μg/ml). In Lane 1, a band at ~100kDa, corresponding to α-ENaC, was detected in the MDCK ENaC lysate (E) but not in the MDCK Parental lane (P). Lanes 3 and 4 are replicates of lanes 1 and 2 respectively. The blot was probed with anti-α-rENaC antibody preincubated with the immunizing peptide. The signal of the 100kDa band detected in Lane 1 was competed by the immunizing peptide (Lane 3). In both blots, primary antibody was detected using a polyclonal goat anti-rabbit-HRP conjugate. The signal was visualized using enhanced chemiluminescence. These experiments are representative of work repeated 3 times.

**Fig. 6. Co-immunoprecipitation of α-rENaC with actin in vivo.**

Alpha-rENaC was immunoprecipitated as described Fig 2A. Lane 1 is the protein G agarose beads control, Lane 2 is the mAb anti-His antibody control and Lane 3 was immunoprecipitated with mAb anti-FLAG antibody for the alpha ENaC subunit. The arrow denotes the detection of actin at approximately 45kDa. All samples were washed with a pH of 9.1. The blot was probed with mAb anti-actin (1:1,500) and the primary was detected using a polyclonal goat anti-mouse-HRP conjugate. The signal was visualized using enhanced chemiluminescence. This experiment is representative of work repeated 3 times.

**Fig. 7. Non-muscle F-actin binds to the GST α-hENaC C-terminal fusion protein in gel overlays.**

Panel A) GST α-hENaC C-terminal fusion (1.0 μg) separated on a 12% SDS-PAGE gel, stained with Gel Code. The arrow indicates the full length fusion protein, and an asterisk indicates degradation products of
the fusion protein. **Panel B**) Lane 1 is GST α-rENaC N-terminal fusion protein as a negative control (1.0 μg), Lane 2 GST negative control (1.0 μg), Lane 3 GST α-hENaC C-terminal fusion protein (1.0 μg), Lane 4 positive control MDCK ENaC whole cell lysate (20 μg), Lane 5 GST γ-rENaC C-terminal fusion protein negative control (1.0 μg). Lane 1 shows very little binding by F-actin/Alexa488. Both GST (lane 2) and GST γ-rENaC C-terminal fusion protein (lane 5) show no detectable F-actin binding. F-actin binds to GST α-hENaC C-terminal fusion protein (lane 3) and to the positive control cell lysate (lane 4). **Panel C** Microtubule and F-Actin/Alexa488 Competition Assay, A single gel lane was loaded with GST α-hENaC C-terminal fusion protein (1.0 μg). Binding of non-muscle F-actin/Alexa488 to GST α-hENaC C-terminal fusion protein was observed. Samples were separated on a 12% SDS PAGE gel, transferred, and blocked overnight in 1X TBST/5% non-fat dry milk. Panel B was probed with non-muscle F-actin/Alexa488 25 μg/ml in 1X TBST/5% non-fat dry milk containing 5 μM phalloidin for 2 h in the dark. Panel C was probed with an equal molar concentration of microtubules and non-muscle F-actin/Alexa488 to determine if the binding of F-actin to the GST α-hENaC C-terminal fusion proteins was specific. Arrow denotes F-actin/Alexa488 conjugate binding to the full length GST α-hENaC C-terminal fusion protein in both Panel B and Panel C. **Panel D** Labeled and Unlabeled F-actin Control, Increased amount of unlabeled non-muscle F-actin decreased binding of labeled non-muscle F-actin/Alexa488. GST α-hENaC C-terminal fusion protein was separated, transferred and blocked as above in Panels B, C. Lane 1 was probed simultaneously with non-muscle F-actin/Alexa488 25 μg/ml and unlabeled non-muscle F-actin 100 μg/ml. Lane 2 was probed with an increased concentration of unlabeled non-muscle F-actin 1 mg/ml while the concentration of labeled non-muscle F-actin/Alexa488 was the same as Lane 1 at 25 μg/ml. The arrow points to full length GST α-hENaC C-terminal fusion protein. The overlay buffer used in Panel D was the same as used in Panels B, C. **Panel E** Non-muscle F-actin binds to 1.0 μg of GST α-rENaC C-terminal fusion protein. The arrow points to full length GST α-rENaC C-terminal fusion protein, and the asterisk indicates a degradation product of this fusion protein. This overlay was done in the same manner as Panel B. Panel A is representative of work repeated 4 times, and Panel B is representative of work repeated 5 times. Panel C is representative of work repeated 3 times, Panel D is representative of work repeated 2 times, and Panel E is representative of work repeated 3 times.

**Fig. 8.** Non-muscle F-actin co-sediments with GST α-hENaC C-terminal fusion protein.

Arrows point to full length GST α-hENaC C-terminal fusion protein, and to the positive control, α-actinin (top right arrow). An asterisk marks degradation products from full length GST α-hENaC C-terminal fusion protein. The majority of GST α-hENaC C-terminal fusion protein was detected in the soluble fraction of the test spin control (no actin). The majority of the GST negative control did not co-sediment with non-muscle F-actin. GST α-hENaC C-terminal fusion protein co-sedimented with non-muscle F-actin. The positive control, α-actinin, was observed to co-sediment with non-muscle F-actin. S=Supernatant, P=Pellet. Each S & P are the soluble and pellet fractions from the same sample. This experiment is representative of work repeated 3 times.
FIGURE 1. Determination of Functional ENaC in stably transfected MDCK ENaC cell line.

A

Basal

B

2 μM Amiloride

C

Real Time Record

2 pM Amiloride

260 pA

25 seconds

D

Current inhibited by Amiloride

N=3

E

ENaC-Transfected MDCK; Basal

ENaC-Transfected MDCK; cAMP

F

Outside-out Patch (100 nM amiloride)
FIGURE 2. Localization of α-rENaC in MDCK ENaC cell line.
FIGURE 3. Localization of F-actin with respect to the apical plasma membrane in MDCK ENaC cells.
FIGURE 4. Co-localization of F-actin and α-ENaC in stably transfected MDCK cells.
FIGURE 5. Immunoprecipitation of α-rENaC from MDCK rENaC whole cell lysates.

A. 
| IP: FLAG | IB: FLAG | IP: FLAG | IB: GAM-HRP |
|----------|----------|----------|-------------|
| Beads    | MsrG     | FLAG     | Beads       |

B. 
| IP: FLAG | IP: FLA | IP: FLAG |
|----------|----------|----------|
| IB: α rENaC | IB: α rENaC & Blocking Peptide |

FIGURE 6. Co-immunoprecipitation of α-rENaC with actin in vivo.

pH 9.1 Wash

| kDa | Beads | His | FLAG |
|-----|-------|-----|------|
| 75  |       |     |      |
| 50  |       |     |      |
| 37  |       |     |      |

Actin
FIGURE 7. Non-muscle F-actin binds to the GST α-hENaC C-terminal fusion protein in gel overlays.

GST-αhENaC C terminus
FIGURE 8. Non-muscle F-actin co-sediments with GST α-hENaC C-terminal fusion protein.
The carboxy terminus of the α-subunit of the amiloride-sensitive epithelial sodium channel (ENaC) binds to F-actin
Christopher Mazzochi, James K. Bubien, Peter R. Smith and Dale J. Benos

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