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Localization of Epithelial Sodium Channels by Atomic Force Microscopy

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1. Introduction

Epithelial sodium channels (ENaC) mediate Na reabsorption across a variety of sodium reabsorbing epithelia, such as the kidney, distal colon, and airway. Normal function of these channels is critical for processes as diverse as blood volume control and airway fluid homeostasis. The molecular cloning of ENaC from a variety of epithelial cells has revealed that they are composed of three homologous subunits, such as α, β, and γ ***(1)***.

Each subunit consists of intracellular N and C termini, two membrane transmembrane domains, and a large extracellular loop (**Fig. 1**; **ref. *1***).

We have previously used atomic force microscopy (AFM) for high-resolution imaging of the apical distribution of endogenously expressed ENaC in *Xenopus* A6 renal epithelial cells ***(2)***. A6 cells are a well-characterized and widely used model of a Na^+ reabsorbing epithelium. A6 cells were grown on cover slips and surface labeled with an antibody generated against an epithelial sodium channel complex purified from bovine renal medulla that had been coupled to 8-nm colloidal gold particles before preparation for AFM ***(2)***. We were successfully able to image ENaC on the cell surface of intact cells because the antibody recognized an extracellular epitope in the channel complex. However, this antibody is no longer available and the anti-ENaC antibodies that are currently commercially available have been generated against intracellular (N and/or C termini) epitopes of the subunits. Insertion of epitope tags into the extracellular domains of transmembrane proteins is a widely used approach for analysis of the cell surface distribution of heterologously expressed transmembrane proteins. Here we describe methods that can be used for AFM imaging of the cell surface distribution of heterologously expressed epitope tagged ENaC.

From: *Methods in Molecular Biology, vol. 242: Atomic Force Microscopy: Biomedical Methods and Applications*
Edited by: P. C. Braga and D. Ricci © Humana Press Inc., Totowa, NJ

2. Materials

1. α, β, and γ ENaC subunit cDNAs in a mammalian expression vector such as pcDNA3.1 (Invitrogen).
2. Standard molecular biological equipment including thermal cycler and agarose and sequencing gel equipment.
3. Gold chloride ($HAuCl_4$).
4. Trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$).
5. Tannic acid.
6. Bovine serum albumin (IgG free).
7. Polyethylene glycol.
8. Sodium azide.
9. Anti-FLAG monoclonal antibody (M2-Sigma) or anti-HA monoclonal antibody (Roche, 3F10).
10. Control mouse IgG (Jackson Immunoresearch; West Grove, PA).
11. Lipofectamine 2000 (Invitrogen), FuGENE 6 (Roche), or a similar cationic lipid transfection reagent.
12. HEK 293 cells (ATCC # CRL-1573).
13. Thermanox cover slips (13 mm in diameter; Nunc).
14. Serum-free media for transfection, such as OPTI-MEM 1 (Gibco-BRL).
15. Amiloride.
16. Glutaraldehyde.
17. Sodium cacodylate.

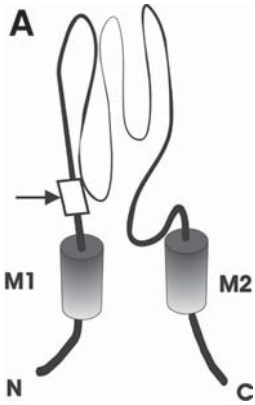
3. Methods

The methods described below outline: (1) the construction of epitope-tagged ENaC plasmids, (2) preparation of colloidal gold-anti-epitope tag antibody conjugates, (3) heterologous expression of epitope-tagged ENaC, (4) antibody labeling of ENaC expressing cells, and (5) AFM imaging of ENaC-expressing cells.

3.1. Construction of Epitope-Tagged ENaC Plasmids

To allow detection of ENaC expressed at the cell surface, the α, β, and γ ENaC subunits are tagged in their extracellular loops at the regions shown in **Fig. 1** with either the FLAG peptide (DYKDDDDK) or the HA peptide (YPYDVPDYA). These regions were initially chosen because they show a high degree of sequence divergence between ENaC subunits of *Xenopus*, rat, mouse, and human ***(3)***. Insertion of either the FLAG or HA epitope tag into these regions does not affect channel assembly or function ***(3,4)***.

Introduction of FLAG or HA peptides into the extracellular loops of α, β, and γ ENaC (*see* **Note 1**) is performed by polymerase chain reaction-based methods as described by Ausbel et al. ***(5)***. In α and γ ENaC, this involves the replacement of amino acids in the extracellular loop with the FLAG or HA epitope and in β ENaC, it involves the insertion of the FLAG or HA epitope



B

α

	Replacement	**DYKDDDDK/ YPYDVPDYA**
α rat/mouse ENaC	^{191}NSSY**TRQAG(A/G)RR**RSSR^{205}	
α human ENaC	^{165}SSFT**TLVAGS . .** RSRR^{178}	
α *Xenopus* ENaC	^{129}NGTG**VQGWIPNN**QRVK^{144}	

β

	Insertion	**DYKDDDDK/ YPYDVPDYA** ↓
β rat/mouse ENaC	^{135}NT . . . . . T(S/N)TLN^{141}	
β human ENaC	^{135}NA . . . . . TRNLN^{141}	
β *Xenopus* ENaC	^{141}NTFHNNQTRQNVTLVD^{156}	

γ

	Replacement	**DYKDDDDK/ YPYDVPDYA**
γ rat/mouse ENaC	^{139}EAGS**MPSTLEGT**PPRF^{154}	
γ human ENaC	^{139}EAES**WNSVSEGK**QPRF^{154}	
γ *Xenopus* ENaC	^{140}KRDV**GVNVENST** EDI F^{155}	

Fig. 1. Placement of the FLAG and HA epitope into the extracellular domains of α, β, and γ ENaC. **(A)** Schematic diagram of an ENaC subunit illustrating the region (arrow) of the extracellular domain that is modified by the replacement or insertion of the epitope tag. **(B)** Sites of replacement (α and γ subunit) and insertion (β subunit) of the FLAG (DYKDDDDK) or HA (YPYDVPDYA) epitope tags into the amino acid sequence of the extracellular domains of ENaC subunits. The nine amino acids that are replaced in α and γ subunits are indicated in bold. For insertion of the HA eptiope, an additional amino acid (underlined) is replaced. The site of insertion of the epitope tag into the β subunit is indicated by the arrow. Based upon **ref. *3***.

into the extracellular loop as shown in **Fig. 1**. The accuracy of the constructs must be verified by restriction enzyme digestions and DNA sequencing. If the

ENaC subunit cDNAs are not in a mammalian expression vector, the cDNAs can be subloned into a suitable expression vector, such as pcDNA3.1, using standard molecular biological techniques. The constructs should be verified by restriction enzyme digestions and DNA sequencing before their expression is attempted.

3.2. Preparation of Colloidal Gold Anti-Epitope Tag Antibody Conjugates

Putnam and coworkers originally described the use of immunogold labels as cell surfaces markers in atomic force microscopy ***(6)***. We have found that 8-nm colloidal gold particle antibody conjugates work well for the detection of ENaC at the cell surface by AFM ***(2)***. Below, we describe the preparation of 8-nm colloidal gold particles after the tannic acid methods of Slot and Geuze (*see* **Note 2**; **ref. *7***) and the conjugation of the colloidal gold particles to anti-eptiope tag antibody or control IgG.

1. Prepare solution A consisting of 1 mL of 1% $HAuCl_4$ and 79 mL of distilled water.
2. Prepare solution B consisting of 4 mL of 1% $C_6H_5Na_3O_7 \cdot 2H_2O$, 15.5 mL of distilled water, and 0.25 mL of 1% tannic acid.
3. Warm solutions A and B to 60°C, mix rapidly, and heat with stirring until boiling.
4. Stabilize the conjugates by the addition of polyethylene glycol to a final concentration of 0.5% before adjusting the pH of the gold solution to 9.0 with 200 m*M* K_2CO_3. It is critical that the solution be stabilized before adjusting the pH because the unstabilized gold solution will destroy the pH electrode.

Next, the colloidal gold particles should be conjugated to the anti-eptiope tag antibody or control IgG following the protocol of Hartwig ***(8)***.

1. Dialyze anti-epitope tag antibody (anti-FLAG epitope or anti-HA epitope monoclonal antibody) and matched control mouse IgG (approximate concentration of 1 mg/mL) against 2 m*M* $Na_4B_4O_7$, pH 9.0.
2. Add either anti-epitope tag antibody or control IgG (120 µg) to 20 mL of stabilized colloidal gold solution, pH 9.0 and rapidly stir for 20 min.
3. Stabilize conjugates by the addition of 250 µL of 8% bovine serum albumin (IgG free) and 20 µL of 5% polyethylene glycol.
4. Collect colloidal gold IgG conjugates by centrifugation at 50,000*g* for 1 h (4°C).
5. Resuspend pellet in 1 mL of 150 m*M* NaCl, 20 m*M* Tris, 1% bovine serum albumin, and 0.1% sodium azide, pH 8.3, and remove aggregates by centrifugation for 10 min at full speed in a microcentrifuge
6. Store conjugates at 4°C. They remain stable for several months.

3.3. Heterologous Expression of Epitope-Tagged ENaC

Next, the epitope-tagged ENaC subunits are transiently expressed in a well-characterized mammalian cell line, such as HEK 293 cells (*see* **Note 3**).

1. HEK 293 cells to be transfected are plated on a Thermanox circular cover slip placed in either a 35-mm tissue culture dish or a single well of a 6-well tissue culture dish. The day before transfection, trypsinize and count to determine the plating density. Cells should be 80–90% confluent on the day of transfection. Plate cells in normal growth medium containing serum but without antibiotics.
2. Transfect cells with epitope tagged α, β, and γ ENaC (start with 0.3 μg of each plasmid/cover slip) or an equivalent concentration of empty vector to serve as a control following the manufacturer's directions for the cationic lipid transfection reagent chosen (i.e., Lipofectamine 2000, FuGENE 6; *see* **Note 4**). Use a serum-free medium, such as OPTI-MEM 1, for the transfection procedure. Replace the media containing the transfection complexes 3–6 h after transfection with fresh complete media (containing serum and antibiotics). Supplement the media with 10 μ*M* amiloride to prevent cell swelling and lysis as a result of ENaC expression.
3. Cells are ready for antibody labeling 24–48 h after transfection.

3.4. Antibody Labeling of ENaC-Expressing Cells

The next step in the process involves the labeling of the transfected cells with the antibody colloidal gold conjugates.

1. Wash cover slips bearing transfected cells in phosphate-buffered saline (PBS) 1 m*M* $CaCl_2$, 3 m*M* KCl, 1 m*M* K_2HPO_4; 2 m*M* $MgCl_2$, 140 m*M* NaCl, 8 m*M* Na_2HPO_4; pH 7.4 (2 × 5 min).
2. Depending upon the epitope tag used, incubate cover slips in either colloidal gold conjugated anti-FLAG or anti-HA tag antibody diluted in PBS for 45 min at 4°C to prevent internalization of the colloidal gold conjugates. Also incubate cover slips bearing transfected cells in colloidal gold-control IgG conjugates diluted in PBS to serve as controls. As additional controls, incubate cover slips bearing nontransfected cells and cells transfected with vector only in colloidal gold antibody and colloidal gold IgG conjugates. The optimal dilution of the colloidal gold antibody complexes will need to be determined for each conjugation. A suggested range of dilutions is 1:10 to 1:100.
3. Wash cover slips in PBS (4 × 5 min) at 4°C and then fix cells for 15 min in 0.25% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.5.
4. Wash cover slips in PBS (2 × 5 min). Cells are now ready for AFM imaging.

3.5. AFM Imaging of ENaC-Expressing Cells

Here, we briefly describe AFM imaging of the cells using a Nanoscope III (Digital Instruments) equipped with the "D" scanner (maximal *x*, *y* scan size 14 mm) and cantilevers with a spring constant of 0.6 N/m and estimated tip diameter of 10 nm (Digital Instruments).

1. Attach a cover slip to the metal AFM puck using double-sided adhesive tape and mount in the fluid cell. Image the sample in PBS.

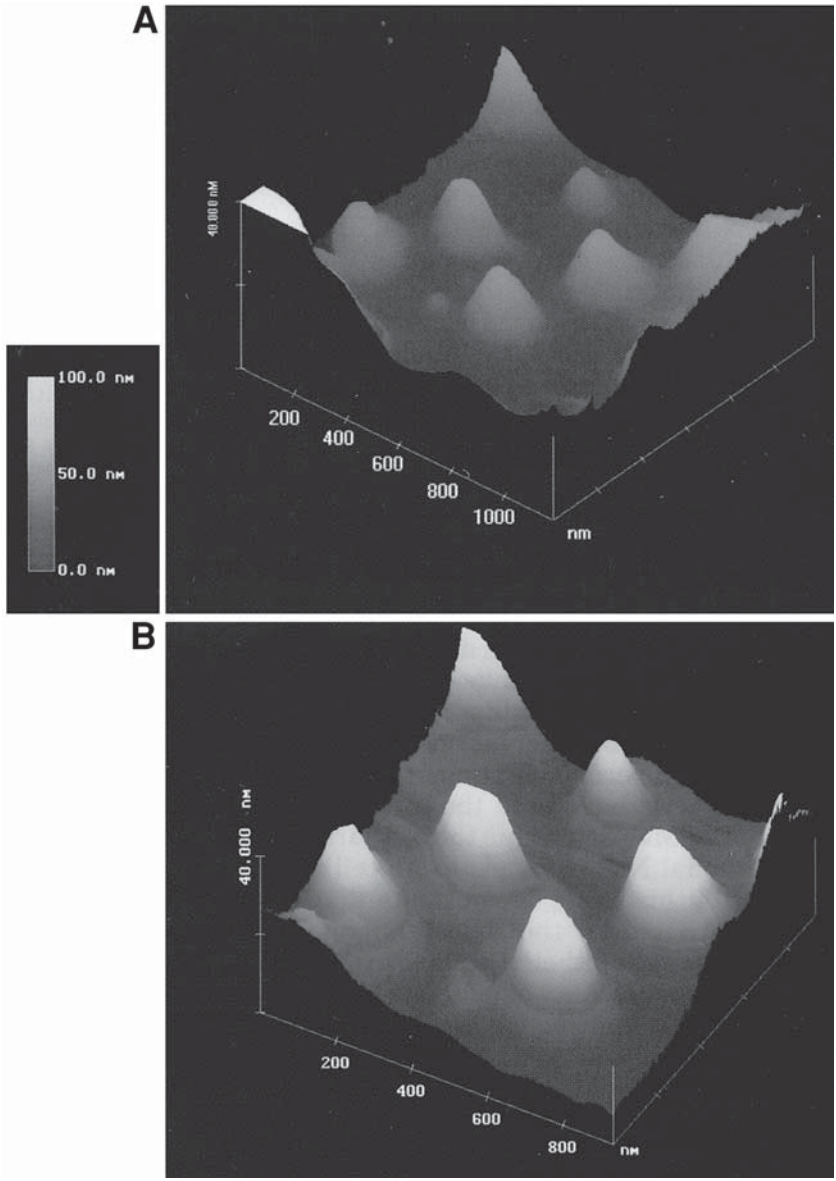


Fig. 2. Representative images illustrating the localization of epithelial sodium channels by AFM. **(A)** Image of the surface of an A6 renal epithelial cell labeled with colloidal gold particles conjugated to nonimmune IgG showing the microvilli. **(B)** Image of the surface of an A6 cell labeled with colloidal gold particles conjugated to an anti-epithelial sodium channel antibody showing localization of marker to the microvilli. Note marked increase in height of microvilli when compared to A. Reproduced with permission from the American Physiological Society from **ref. *2***.

2. Operate Bioscope in the contact mode. After engagement with the sample, adjust the scan force to values in the range of 5 nN. Record images with a display of 512 lines/screen (1 μm^2 scan area) at a constant force. (Typical area rate of 1–2 Hz.)
3. Scan multiple cells/cover slip and multiple cover slips labeled with both antibody and control IgG. Also scan cover slips bearing cells that were fixed but not labeled to obtain dimensions and surface topography of the cells before antibody treatment. Binding of colloidal gold antibody conjugates to the epitope-tagged ENaC results in a marked difference in height of the cell surface when compared to controls (*see* **Fig. 2**; **Note 5**).

4. Notes

1. cDNAs have been cloned for the α, β, and γ subunits of *Xenopus* ***(9)***, rat ***(10–13)***, mouse ***(14)***, and human ENaC ***(15–17)***. All species can be expressed heterologously in mammalian cells. Typically, the cDNAs are available upon request from the laboratories that cloned them. Subcloning of the subunits into a mammalian expression vector, such as pcDNA3.1, may be required. A number of laboratories have produced constructs for ENaC subunits with extracellular epitope tags that may be available upon request ***(3,4,18)***.
2. Although we produce colloidal gold particles following the method of Slot and Geuze ***(7)***, colloidal gold particles produced by this method are available commercially (Sigma; Electron Microscopy Sciences, Fort Washington, PA).
3. HEK 293 cells have been effectively used for the transient expression of ENaC ***(19,20)***. When HEK 293 cells are used with an expression vector that includes the CMV promoter, such as pcDNA 3.1, high levels of transcription are obtained. COS 7 cells ***(19)*** and Fisher rat thyroid cells, which form polarized monolayers ***(21)***, have also proven useful for the transient expression of ENaC. Alternatively, stably transfected cell lines expressing epitope-tagged α, β, and γ ENaC can be generated ***(18,20,22)***. The selection of an appropriate cell line and the use of transient or stable transfectants depends upon the objectives of the investigation.
4. For transient tranfection using liposome-mediated transfection reagents, we suggest starting with a plasmid concentration of 0.3 μg for each ENaC subunit. This, however, will need to optimized in each lab. Follow the manufacturer's directions for optimization of transfection efficiency and protein expression levels. To achieve high efficiency transfections, it is critical that the plasmid DNA used is of high quality and is free of contaminants.
5. For visualization of colloidal gold antibody conjugate on the cell surface, select small areas of interest during scanning and reduce the field to concentrate on these areas. In addition, small areas of interest can be selected from stored images, zoomed to full screen and analyzed using the Nanoscope III software. To control for bias during both scanning and analysis, it is recommended that a blind study be performed.

Acknowledgments

This work was supported by National Institutes of Diabetes and Digestive and Kidney Diseases Grants DK-37206 (Dale J. Benos) and DK-56596 (Peter

R. Smith). Peter R. Smith is the recipient of an Established Investigator Award from the American Heart Association.

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