A Region Directly Following the Second Transmembrane Domain in γENaC Is Required for Normal Channel Gating*

Received for publication, May 22, 2003, and in revised form, August 1, 2003
Published, JBC Papers in Press, August 3, 2003, DOI 10.1074/jbc.M305400200

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We used a yeast one-hybrid complementation screen to identify regions within the cytosolic tails of the mouse α, β, and γ epithelial Na+ channel (ENaC) important to protein-protein and/or protein-lipid interactions at the plasma membrane. The cytosolic COOH terminus of αENaC contained a strongly interactive domain just distal to the second transmembrane region (TM2) between Met110 and Val132. Likewise, γENaC contained such a domain just distal to TM2 spanning Gln573–Pro600. Interactive domains were also localized within Met1–Gln17 and the last 17 residues of α- and βENaC, respectively. Confocal images of Chinese hamster ovary cells transfected with enhanced green fluorescent fusion proteins of the cytosolic tails of mENaC subunits were consistent with results in yeast. Fusion proteins of the NH2 terminus of αENaC and the COOH termini of all three subunits co-localized with a plasma membrane marker. The functional importance of the membrane interactive domain in the COOH terminus of γENaC was established with whole-cell patch clamp experiments of wild type (α, β, and γ) and mutant (α, β, and γγQ573P600) mENaC reconstituted in Chinese hamster ovary cells. Mutant channels had about 13% of the activity of wild type channels with 0.33 ± 0.14 versus 2.5 ± 0.80 nA of amiloride-sensitive inward current at −80 mV. Single channel analysis of recombinant channels demonstrated that mutant channels had a decrease in Popen with 0.16 ± 0.03 versus 0.67 ± 0.07 for wild type. Mutant γENaC associated normally with the other two subunits in co-immunoprecipitation studies and localized to the plasma membrane in membrane labeling experiments and when visualized with evanescent-field fluorescence microscopy. Similar to deletion of Gln573–Pro600, deletion of Gln573–Arg583 but not Thr580–Pro586 decreased ENaC activity. The current results demonstrate that residues within Gln573–Arg586 of γENaC are necessary for normal channel gating.

Activity of integral membrane proteins is regulated, in general, by the following two means: post-translational modification, and discretion and interaction with accessory, regulatory proteins and/or lipids. These two modalities of regulation are not necessarily mutually exclusive and impact function by influencing several parameters, including protein localization and kinetics. Ion channels are integral membrane proteins that play fundamental roles in many diverse cellular processes. Similar to other membrane proteins, ion channel activity is, in part, a manifestation of channel kinetics and cellular locale.

The amiloride-sensitive epithelial Na+ channel (ENaC) is an ion channel localized to the luminal plasma membrane of epithelial cells (1–3). Activity of this channel is the rate-limiting step in Na+ transport across electrically tight epithelium. Thus, ENaC plays a pivotal role in Na+ and concomitant water (re)absorption across many epithelial tissues. This channel, consequently, is centrally positioned as an effector for systemic hormones and other factors that modulate blood pressure. Gain and loss of function mutations in ENaC and its regulatory pathways, indeed, cause blood pressure disorders in humans associated with aberrant Na+ and water metabolism (4). Although it is accepted that ENaC activity is dynamically modulated by regulation of channel localization to the luminal membrane, little is actually known about the cellular control points and queues impinging upon this modulation. In addition, the specific residues and domains within the channel itself important to localization and control of channel activity remain obscure.

ENaC is a member of the Deg/ENaC superfamily of ion channels (3, 5). This superfamily contains a functionally diverse array of channels that all share a common tertiary structure with members having two-transmembrane spanning regions, a large extracellular ectodomain and two short cytosolic tails. Channels within this superfamily play important roles in sensory perception, including taste, touch, hearing, nociception, and neurotransmission, as well as vectorial Na+ transport across epithelia. In native epithelia, ENaC is composed of three homologous but distinct subunits: α, β, and γ. Canessa et al. (6, 7) and Lingueglia et al. (8) were the first to identify the molecular correlates of ENaC. Most results suggest that the functional channel has a stoichiometry of two α and one β and γ subunit (9, 10); however, the alternative that the functional channel is composed of three copies of each of the three subunits has also been proposed (11). Heterologously expressed αENaC alone and together with either β- or γENaC also forms homomeric and heterodimeric channels, although with much de-
creased activity and slightly different biophysical characteristics from the endogenous channel in native epithelia (6, 7, 12–14).

The cytosolic tails of ENaC are believed to be regulatory domains and/or effecter sites that impinge on channel gating and locale. Recent findings from our laboratory showing that the NH$_2$ terminus of αENaC and the COOH termini of all three subunits contain domains involved in protein-protein and/or protein-lipid interactions localized to the plasma membrane are consistent with such a possibility (15). Moreover, deletion of the entire NH$_2$ terminus from any subunit inactivates ENaC (16). Conversely, deletion of the complete COOH tail of β- and γ-ENaC but not αENaC activates the channel (17, 18). Deletion of the latter half of the COOH tail of αENaC, however, does increase activity (19). These results, as well as others, suggest that the cytosolic tails of ENaC are involved in both positive and negative regulation of channel activity. Interestingly, the COOH terminus of ENaC subunits are the least well conserved portions of the channel. It has been hypothesized that these regions may impart the well described tissue- and species-specific regulation of ENaC by allowing differential interaction with tissue-specific intermediary effector/accessory proteins (5).

The most well described regulation of ENaC involving the cytosolic tails of the channel is down-regulation of activity upon binding of the ubiquitin ligase Nedd4. The WW domains within Nedd4 target this protein and similar ligases to PY motifs (XPPXY) in the distal portions of the ENaC COOH termini promoting ubiquitination of the NH$_2$ terminus of α- and γENaC subunits and subsequent internalization of the channel (20, 21). The cytosolic COOH tails of αENaC also contain a tyrosine-based endocytic tag overlapping the PY motif (YXXL) (22) that in some instances is functionally independent of the PY motif at least in γENaC (15). Moreover, COOH tails contain SH3 binding domains (23). Such a domain in αENaC binds the SH3 domain within α-spectrin and has been implicated in localizing the channel to the luminal membrane in epithelia. In addition, the COOH termini of β- and γENaC may impact ENaC open probability by promoting channel closing (24, 25). All of these COOH-terminal domains, described previously (15), are more distal than the membrane reactive domain we recently identified in the COOH tail of γENaC.

The NH$_2$ terminus of αENaC is required for normal channel function (16). Overexpression of a peptide containing this region of αENaC acts as a competitive inhibitor of wild type channels. Channels missing the first 109 residues of αENaC increases conductance (24). The COOH terminus of αENaC acts as a competitive inhibitor of wild type channels. Channels missing the first 109 residues of αENaC, in addition, have decreased activity; however, they localize to the plasma membrane (16, 26). This region also contains another possible endocytic tag (KGDK) (16) and a well conserved 5-amino acid tract containing a glycine (Gly$_95$) crucial to normal channel gating (27). Interestingly, α-rENaC is differentially spliced to produce αENaC subunits with unique NH$_2$ termini (28). The functional ramifications of this have yet to be determined.

Similar to the NH$_2$ terminus and to the COOH terminus of the other subunits, the COOH terminus of αENaC plays a role in modulating channel activity. Binding of actin to the COOH tail of αENaC increases channel open probability but decreases conductance (24). The COOH terminus of αENaC also contains a region that supports channel activity and is involved in kinase regulation of the channel (19). In particular, residues Pro$_{505}$ and Gly$_{506}$ in αENaC are critical to normal localization of the channel to the plasma membrane.

Thus, there is convincing evidence that the cytosolic tails of ENaC subunits affect channel activity by impacting both channel locale and gating. However, only a few residues and specific domains within these regions of ENaC have been identified in detail and linked to function. Gupta and Canessa (29) reported previously that heterologous expression of α- and β-rENaC results in yeast becoming salt- and amiloride-sensitive, demonstrating that this recombinant channel is active in this background. In the current study, we built on this earlier work by
using a simple yeast one-hybrid complementation screen to define regions within the cytosolic tails of ENaC important to protein-protein and/or protein-lipid interactions at the plasma membrane. Importantly, we determined that a region within the COOH-terminal tail of βENaC identified with our yeast screen had functional ramifications in a mammalian system.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of reagent grade and purchased from either Sigma or Fisher unless noted otherwise. The BCA Protein Assay was from Pierce. All materials used in Western blot analysis were from Bio-Rad. The monoclonal anti-Myc antibody was from Clontech (Palo Alto, CA) and anti-HA was from Roche Applied Science. Anti-mouse horseradish peroxidase-conjugated 2° antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). ECL reagents were from PerkinElmer Life Sciences. All DNA sequencing was performed by the molecular biology core facility at the University of Texas Health Science Center, San Antonio. The Saccharomyces cerevisiae cdc25H yeast strain (cdc25H: MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-90 leu2-3,112 cdc25-2 (ts) Gal+) and the pSOS plasmid were from Stratagene (La Jolla, CA). The pECFP-M, pEGFP-F, pDsRed2-N, pCMV-Myc, and pCMV-HA plasmids were from Clontech. The plasmids encoding mouse ENaC subunit cDNAs have been described previously (30) and were the gift from Dr. T. R. Kleyman.

Plasmodiaceae—Full-length mouse α-, β-, and γENaC were ligated in-frame behind the epitope tag into pCMV-Myc and pCMV-HA by using XhoI and NolI. Initially, channel subunits were amplified from the original pBlueScript (SK−) plasmids described by Ahn and colleagues (30) with standard PCRs. For α-, β-, and γ-ENaC, the upstream and downstream primers were 5′-CGAATTCGATTATGCGACACCAC- CAGAAC and 5′-GCAAGGGCGCCCTGACTGCACCTGCGCAGGAGCACG; 5′-CGAATTCGATTATGCGACACCACAGACAGGGCCCACTGGCGGC upstream and downstream primers, respectively, in conjunction with full-length pCMV-myc-ENaC used to create hSOS fusion proteins are noted. B, a typical screen is shown. The top source plate developed at permissive temperature (24°C) contained Cdc25 yeast transformed with hSOS-βENaCtail hybrids. The middle and lower plates contain parallel arrays of colonies patched from the respective source plate developed at permissive (middle) and restrictive (37°C; bottom) temperatures. Complementation was graded positive if colonies developed at restrictive temperatures.

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Conical Imaging—Transfected CHO cells were grown on number 0 coverslips, fixed in 4% paraformaldehyde, and mounted using Vectashield (Vector Laboratories, Burlingame, CA). Confocal images were collected using an ×60 (1.3 NA) oil-immersion lens on a Nikon Eclipse TE2000 (Nikon Instruments, Melville, NY) inverted microscope fitted with a Cascade Photometric CCD camera (Roper Scientific, Tucson, AZ), the CARV confocal fluorescence imaging unit (Kinetic Imagine, Weston, Ontario, Canada), and a Lambda 10-2 filter wheel (Sutter Instruments, Novato, CA). This unit is driven by the Metamorph program suite (Universal Imaging Corp., Downingtown, PA) and faced with a piezosystem (Piezosystem Jena, Hopedale, MA) per the manufacturer’s recommendations. In brief, cells ~60% confluent in a 35-mm dish were treated with 2.5 μg of total plasmid cDNA for 24–48 h. Cells were used for patch clamp analysis up to 96 h after transfection and were maintained in culture in the presence of 10 μM amiloride replenished daily. Cells used for protein analysis were grown in 100-mm dishes, transfected with 4 μg of total plasmid cDNA, and extracted 24–48 h after transfection.

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In conjunction with a single pass excitation filter. With these filter sets, fluorophores were easily discriminated with no bleed through (see Fig. 2).

Evanescent-field (EF) Fluorescence Microscopy—To selectively illuminate the plasma membrane and its associated channel subunits, we used EF microscopy. Cells used for EF microscopy were plated on glass coverslips and fixed as above for confocal imaging. Methods followed closely those described previously by Almers and colleagues (32, 33). In brief, EF microscopy was performed using an inverted TE2000 microscope with through-the-lens fluorescence imaging. EF illumination was generated by total internal reflection fluorescence (TIRF) after the light beam struck the interface between the glass coverslip and cellular plasma membrane at a glancing angle (34). Samples were viewed through a Plan Apo TIRF ×60 oil-immersion, high resolution (1.45 NA) objective (Nikon). TIRF generates an EF that declines exponentially with increasing distance from the interface between the coverglass and cellular plasma membrane at a glancing angle (34). Samples were viewed through a Plan Apo TIRF ×60 oil-immersion, high resolution (1.45 NA) objective (Nikon). TIRF generates an EF that declines exponentially with increasing distance from the interface between the cover glass and plasma membrane illuminating only a small optical slice of the cell (∼200 nm) including the plasma membrane. Thus, with TIRF only fluorophores in the plasma membrane and its immediate vicinity contribute to emission, whereas those deeper in the cell do not (see Fig. 8A). DsRed2 and EGFP-F were excited with green HeNe and argon lasers, respectively, with emissions subsequently passing through 543- and 488-nm single pass filters, respectively. This system was also interfaced with a mercury lamp with appropriate dichroic excitation and emissions filter sets enabling wide field epifluorescence imaging of DsRed2 and EGFP-F. Images were collected and processed as above with a CCD camera interfaced to a PC running Metamorph software.

Patch Clamp Recording and Single Channel Analysis—Whole-cell macroscopic current recordings of ENaC reconstituted in CHO cells were made under voltage clamp conditions using standard methods (31). Prior to patch clamp analysis, cells were rinsed of culture media and amiloride and patched at room temperature under constant perfusion in a bath solution of (in mM) 160 NaCl, 1 CaCl₂, 2 MgCl₂, and 10 HEPES (pH 7.4, 320 mOsm). Pipette solution was (in mM) 145 KCl, 5 NaCl, 2 MgCl₂, 0.5 CaCl₂, 10 EGTA, 10 HEPES (pH 7.4), 3.0 ATP, and 0.1 GTP (330 mOsm). Current recordings were acquired with a PC-505B patch clamp amplifier (Warner Instruments; Hamden, CT) interfaced via a Digidata 1320A (Axon Instruments, Union City, CA) with a PC running the pClamp 8.1 suite of software. All currents were filtered at 1 kHz. Both a family of test pulses stepping by 20-mV increments (500 ms each separated by 400 ms) form ∼120 to +100 mV, and voltage ramps (100 ms) over the same range were used to generate current-voltage (I-V) relations. The whole-cell capacitance was routinely compensated and was approximately 12 picofarads for CHO cells. Series resistances, on average 2–5 megohms, were also compensated. Currents, however, were not leak-corrected. For all experiments, holding potential was 30–50 mV. For voltage steps, steady state whole-cell currents were routinely measured 100–200 ms from the start of each pulse.

Single channel current recordings were performed as described previously (35–37). In brief, all experiments were performed at room temperatures with fire-polished pipettes of borosilicate glass (World Precision Instruments, Sarasota, FL) with tip resistances 4–7 megohms. All recordings were made in excised, outside-out patches with pipette and
bath solutions of (in mM) 120 CaCl2, 5 NaCl, 2 MgCl2, 3 ATP, 0.1 GTP, 5 EGTA, 10 HEPES (pH 7.3), and 160 NaCl, 1 CaCl2, 2 MgCl2, 10 HEPES (pH 7.4), respectively. Inward currents (cystosol to pipette) are shown as downward deflections. All experiments were acquired using pClamp8.1 software with time and current amplitude data analyzed with this software in conjunction with Igor Pro 4.0 (Wavemetrics Inc., Lake Oswego, OR). Single channel unitary current (i) was determined from the best-fit Gaussian distribution of all-point amplitude histograms. Channel activity (NP) was NP = i/I, where I is the mean total current in the patch and i is unitary current at this voltage (calculated from all-point amplitude histograms). By definition then, current at the closed state is 0. Where appropriate, open probability (P_o) was calculated by normalizing NP, for the total number of estimated channels (N) in the patch as described previously (35).

Expression and Electrophysiology in FRT Epithelia—Function of wild type and mutant ENaC expressed in Fischer rat thyroid (FRT) cells was assayed with standard methods described previously (38, 39). In brief, transfected cells (0.07 μg of each α-, β-, and γ-mENaC) were maintained on permeable filter supports. Na+ transport was measured 2–3 days after transfection at 37 °C in modified Ussing chambers (Warner Instrument Corp.) with luminal and serosal solutions of (in mM) 135 NaCl, 1.2 CaCl2, 1.2 MgCl2, 2.4 K2HPO4, 0.6 KH2PO4, 10 dextrose, 10 HEPES (pH 7.4; bubbled with O2). Amiloride-sensitive short-circuit current was determined as the difference in current with and without amiloride (10 μM) applied to the luminal bath solution. Western blot analyses—Western blot analysis of Myc- and HA-tagged ENaC was performed using standard procedures described previously (31, 37, 40, 41). In brief, cells were lysed in gentle lysis buffer, cleared, normalized for total protein concentration, suspended in Laemmli sample buffer and 20 mM DTT, run on 7.5% polyacrylamide gels cleared, normalized for total protein concentration, suspended in Laemmli sample buffer and 20 mM DTT, run on 7.5% polyacrylamide gels in the presence of SDS, transferred to 0.45-μm polyvinylidene fluoride membranes and probed with antibody in Tris-buffered saline supplemented with 5% dried milk (Nestle, Solon, OH) and 0.1% Tween 20. Primary antibody was applied overnight at 4 °C. Blots next were probed with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were developed with enhanced chemiluminescent reagents and Kodak BioMax Light-1 film. Digital images of Western blots were generated using a ScanJet4200C (Hewlett-Packard, Houston, TX).

Immunoprecipitation—CHO cells transfected with epitope-tagged ENaC were extracted in gentle lysis buffer. Whole-cell lysates (400 μl at 1 μg/μl total protein) were treated with anti-Myc-agarose or anti-HA antibody plus protein A/G PLUS-agarose overnight at 4 °C. Antibody plus protein A/G PLUS-agarose was then washed 3 times with ice-cold PBS (pH 8.0) and subsequently applied overnight at 4 °C with 50 μl of gentle lysis buffer and resuspended in sample buffer. Proteins were visualized by Western blotting as described above.

Biotinylation—CHO cells grown and transfected as described above for protein analysis. Biotinylation closely followed that described previously by Heda et al. (42, 43). In brief, 48 h after transfection cells were washed 3 times with ice-cold PBS (pH 8.0) and subsequently incubated with 1 mM sulfo-NHS-LC-biotin (in PBS, pH 8.0) for 30 min at 4 °C in the dark. Cells were washed 3 times with ice-cold PBS and extracted in gentle lysis buffer. Pre-equilibrated streptavidin-agarose beads were agitated overnight at 4 °C with 50 μl of total protein. Agarose beads were then washed 6 times with GLB and subsequently resuspended in sample buffer. Proteins were visualized using standard Western blotting described above.

Statistics—Complementation frequency was compared using a z-test on proportions. A p < 0.05 was considered significant. All patch clamp data are presented as means ± S.E. Paired and unpaired data were compared using appropriate t tests. For multiple comparisons, a one-way analysis of variance with the SNK sub-test was used to establish significances. p ≤ 0.05 was considered significant.

RESULTS

A yeast one-hybrid complementation screen was used to identify regions within ENaC involved in protein-protein and/or protein-lipid interactions. Fig. 1 shows results from a typical screen. For this experiment, two hybrids were created: one containing the NH2 terminus and the other the COOH terminus of βENaC fused to truncated hSOS (catalytic domain) incapable of independently localizing to the plasma membrane. Fig. 1A shows the position of these cytosolic tails relative to the ectodomain and transmembrane domains (TM1 and TM2). Hybrids were overexpressed in Cdc25H yeast. Transfected yeast was maintained on a source plate at the permissive temperature of 24 °C (Fig. 1B, top). Colonies were then patched from the source plates to create duplicate arrays with one developed at permissive (Fig. 1B, middle) and the other restrictive (37 °C; Fig. 1B, bottom) temperatures. Shown in Fig. 1B are typical results from yeast transfected with the entire NH2-terminus (βM1,W223, left) and COOH-terminal tails (βASS5-end, right) of βENaC. These results clearly demonstrate that the COOH-terminus of βENaC localizes to the plasma membrane in yeast allowing complementation of growth at restrictive temperatures. The COOH-terminal tails of all three subunits and the NH2-terminal tail of αENaC complemented growth in this assay (see Fig. 3 (15)).

We tested next whether domains identified as membrane-reactive in the yeast screen yielded comparable results in mammalian cells. Fig. 2 shows representative confocal images of CHO cells transfected with a membrane marker (ECFP-M; Fig. 2, middle column) and one of the six different EGFP-ENaC hybrid proteins containing the cytosolic tails of each subunit (1st column). Fig. 2, 3rd column, shows merged images. For this figure, the NH2 terminus of γENaC was overexpressed with a nuclear marker (DsRed2-N; noted with asterisk). These results clearly show that all three of the COOH termini of ENaC subunits co-localize with the membrane marker. Moreover, the NH2 terminus of αENaC, but not β and γ, also overlapped with the membrane marker. These findings in a mammalian expression system are entirely consistent with findings in yeast (see Figs. 1 and 3 (15)). Together they strongly argue that domains within the cytosolic tails of ENaC contain residues involved in protein-protein and/or protein-lipid interactions localized to or near the plasma membrane. Most important, the results in Fig. 2 validated the yeast screen. Because the yeast screen measures only membrane-localized interactions, localization is easier to grade compared with confocal images, which can be influenced by background fluorescence resulting, in part, from immature and cytosolic pools of the hybrid. Thus, our yeast screen may be more sensitive than traditional confocal micro-
copy with respect to identifying domains involved in protein-protein interactions localized to the plasma membrane.

We further defined the domains within ENaC tails interacting at the plasma membrane using the one-hybrid screen. Shown in Fig. 3 is a summary graph of complementation frequency (right) for various truncation and deletion mutants of pSOS-ENaCtail hybrids. The portion of ENaC (Fig. 3, gray rectangles) tested in each construct is noted relative to TM1 or TM2 (black boxes). A membrane interactive domain was localized to the first half (Met1–Gln54) of the NH2 terminus of αENaC. Upon dividing this peptide, both halves complemented suggesting that domains within both regions localized to the membrane. Residues included within Met610–Pro613 in αENaC, which directly follow TM2, contained a domain strongly interactive at the plasma membrane. Similarly, the region from Ala618–Arg623 in γENaC, which also directly follows TM2, contained a domain strongly interactive at the plasma membrane. Residues Cys586–End in βENaC and Thr584–Pro600 in γENaC also contained membrane interactive domains; however, these constructs resulted in punctate growth suggesting a weak interaction that is approaching the sensitivity threshold of the screen.

The functional relevance of the membrane interactive domains in the COOH tail of γENaC was determined by using a reconstituted mammalian expression system. Fig. 4A shows a representative family of currents from CHO cells transfected with (Myc-tagged) α-, β-, and γ-mENαC elicited by test pulses (20-mV increments) from −120 to 100 mV in the absence (left) and presence (right) of 10 μM amiloride. Fig. 4B shows a family of currents elicited by the same voltage steps in untransfected CHO cells in the absence and presence of amiloride. Fig. 4C is a typical whole-cell I-V relation of a CHO cell overexpressing α-, β-, and γ-mENαC. The solid line is the difference. D, a summary graph comparing the macroscopic inward Na+ current at −80 mV for CHO cells in the presence and absence of amiloride. *p < 0.05 versus control.

Typical families of currents elicited by voltage steps in CHO cells transfected with (Myc-tagged) α-, β-, and γQ573P600 mENαC in the absence (left) and presence (right) of amiloride are shown in Fig. 5A. (Note the scale difference with Fig. 4A.) Fig. 5B compares representative I-V relations for wt (solid line) and mt (dashed line) channels in response to voltage ramps. Fig. 5C summarizes a population study of the amiloride-sensitive, macroscopic, inward Na+ current at −80 mV for wt and mt channels with mt channels having significantly less current than wt with 0.33 ± 0.14 compared with 2.5 ± 0.80 nA (n = 7). This was a single-blinded population study of four distinct parallel transfections where the end user did not know which was the experimental group. The inset in Fig. 5C shows that mt γENαC expressed equally as well as wt in CHO cells. Moreover, no overt difference was observed in the frequency of seals that had amiloride-sensitive currents in the wt versus mt groups (f = 0.6).

To determine whether the mutant γENαC subunit lacking...
Gln573–Pro600 was capable of appropriately interacting with $\alpha^1$, $\alpha^2$, and $\gamma^\Delta Q573-P600$ ENaC subunits, we overexpressed HA-tagged $\alpha^1$- and $\alpha^2$-ENaC with Myc-tagged $\gamma^\Delta Q573-P600$ ENaC in CHO cells and performed co-immunoprecipitation studies. Both wt and mt $\gamma$ENaC were tested in these experiments. Shown in Fig. 6 are typical Western blots containing the anti-HA precipitant (top) and whole-cell lysate (bottom) from CHO cells transfected with wt HA-$\alpha^1$, $\alpha^2$-ENaC in addition to Myc-tagged wt ($\gamma^\Delta Q573-P600$) ENaC. The last lane contains pre-precipitant and lysate from cells transfected with Myc-tagged wt $\gamma$ENaC alone. Both blots probed with anti-Myc antibody. These studies clearly showed that mt $\gamma$ENaC is as effective as wt in interacting with $\alpha^1$- and $\gamma^\Delta Q573-P600$ subunits.

We asked next whether mt $\gamma$ENaC was capable of getting to the plasma membrane. Shown in Fig. 7A is a typical Western blot (cut in half) probed with anti-Myc (top) and anti-Fra-2 (bottom) antibodies. These blots contain the pellet and supernatant from streptavidin precipitations of CHO cells overexpressing EGFP or all three Myc-tagged ENaC subunits (noted by gray boxes). After transfection but before lysing, one sample from the ENaC transfection group was briefly treated with sulfo-NHS-LC-biotin at 4°C to label membrane proteins. These results, consistent with the functional studies in Figs. 5, 6, and 9–11, clearly show that exogenous ENaC is in the plasma membrane. The cytosolic protein Fra-2 localized primarily to the supernatant in streptavidin-precipitated biotinylated preparations demonstrating good separation of membrane-labeled protein from cytosolic protein.

Fig. 7B tested whether the Gln573–Pro600 deletion mutant like that of wild type $\gamma$ENaC localized to the plasma membrane. For this representative experiment, Myc-tagged wt and mt subunits were overexpressed with HA-tagged $\alpha^1$- and $\beta$ENaC. Cells were washed (right) or treated with sulfo-NHS-LC-biotin to label membrane proteins (left), followed by lysing and streptavidin precipitation. Precipitated proteins are shown in the top blot. The lower blot contains whole-cell lysate from each group. Blots were probed with anti-Myc antibody. These results show that channels containing the mutant $\Delta$Gln573–Pro600 subunit similar to the wild type $\gamma$ENaC subunit localize to the plasma membrane. These data in conjunction with those in Figs. 5 and 6 showing that the $\Delta$Gln573–Pro600 mt interacts normally with $\alpha^1$- and $\beta$ENaC and expresses at levels comparable with wt $\gamma$ENaC but that channels containing the mt subunit have less activity suggest, then, that the $\Delta$Gln573–Pro600 mutation most likely affects ENaC gating kinetics and not localization to the plasma membrane.
overexpressing Myc-tagged /H9251
the plasma membrane (/1st column; pseudocolored green). Merged images are shown in the last column. These results clearly show, as expected with this technique, that only fluorophores, such as EGFP-F but not DsRed2, localized to or within the vicinity of the plasma membrane (~100 nm) were excited with EF microscopy. For these experiments all parameters, including gain and exposure times, were held constant in both the wide-field and the EF microscopy groups. Upon greatly increasing the gain and exposure time, we could observe a very modest DsRed2 signal with EF microscopy (note shown) most likely resulting from slight penetration into the perimembrane cytosolic region that contained a small percentage of the DsRed2 signal. Fig. 8B shows EF fluorescence (2nd column; pseudocolored green) and wide-field epifluorescence (1st column pseudocolored red) images of CHO cells transfected with HA-α,β-mENaC in addition to either Myc-tagged wild type γENaC (top) or the ΔGln573–Pro600 mutant (bottom). The last column shows merged images. These cells were permeabilized and exposed to anti-Myc antibody and a rhodamine-conjugated 2° antibody. These results clearly show that channels containing wt and mt γENaC subunits are both similarly localized to within ~100 nm of the cover glass-cell interface most likely in the plasma membrane.

Results in Fig. 9 characterize at the single channel level wt ENaC reconstituted in CHO cells. The current traces in Fig. 9A were from an outside-out patch with CsCl in the pipette and NaCl in the bath. The applied command potential is noted for each trace. Inward current is denoted by downward deflections, and the arrows note the closed state. This patch contained at least six channels. Fig. 9B shows the I-V relation and corresponding channel openings (for voltages ranging from −40 to −120 mV) for this type of channel, which was not observed in untransfected cells. This channel had a single channel conductance (~40 to ~120 mV) of 5.8 ps, was Na⁺-selective, and not voltage-gated. Shown in Fig. 9C are all-point histograms for the current trace in Fig. 9A held at ~60 mV. This Na⁺-selective channel had all the hallmarks of ENaC described in other expression systems and in native epithelia (44–47).

Fig. 10 tested whether this Na⁺-selective, 5.8-pS channel was sensitive to amiloride, which is a well described specific inhibitor of ENaC (44–47). Shown in Fig. 10A are single channel currents from an outside-out patch (no applied potential) made on α-, β-, and γ-mENaC transfected cells containing several of these 5.8-pS Na⁺ channels before and after 2 μM amiloride treatment. The currents under the three gray lines are shown at a faster time scale in the insets with I being before amiloride treatment and II and III after treatment. Shown in

A.

| Biotin   |   |   |   |
|----------|---|---|---|
| GFP      |   |   |   |
| myc-α,β,γ-ENaC |   |   |   |
| Pellet   |   |   |   |
| Supernatant |   |   |   |

P: streptavidin
IB: anti-myc

IB: anti-Fra-2

B.

+ biotin

IP: streptavidin
IB: anti-Myc

no biotin

IP: streptavidin
IB: anti-Myc

+ biotin

whole cell lysate
IB: anti-Myc

no biotin

whole cell lysate
IB: anti-Myc

Fig. 6. Mutant ΔGln573–Pro600 γENaC interacts normally with α- and βENaC. These typical Western blots contain HA-precipitant (top) and whole-cell lysate from CHO cells overexpressing HA-α,βENaC in addition to Myc-tagged mt (/1st lane) and wt (2nd lane) γENaC. The 3rd lane contained samples from cells transfected with Myc-tagged wt γENaC alone. Both blots were probed with anti-Myc antibody. IP, immunoprecipitate; IB, immunoblot.

Fig. 7. Mutant ΔGln573–Pro600 γENaC is as effective as wt γENaC in getting to the plasma membrane. A, typical Western blot (cut in half) probed with anti-Myc (top) and anti-Fra-2 (bottom) antibodies containing the streptavidin precipitant and supernatant from CHO cell lysates overexpressing Myc-tagged α-, β-, and γ-ENaC or GFP. One set of cells transfected with α-, β-, and γ-ENaC was treated with sulfo-NHS-LC-biotin to label membrane protein. B, representative Western blots containing the streptavidin precipitant (top) and whole-cell lysate (bottom) from washed (right) and sulfo-NHS-LC-biotin-labeled (left) CHO cells transfected with HA-tagged α- and β-ENaC plus wt γENaC and mt γ44573–14000 ENaC subunits. Blots were probed with anti-Myc antibody. IP, immunoprecipitate; IB, immunoblot.
These patches were held at 0 mV. The lower three traces are from three different CHO cells expressing mt channels. The typical current traces from CHO cells expressing wt and mt ENaC. Inward current containing the mt ENaC.

P11 addition, the unitary current calculated from the all-point histograms in Fig. 11 is 0.41 and mt ENaC transfected cell that contained 57 channels with a Po of 0.57 and mt Gln573––Pro600 mutants. Fig. 13 shows representative currents elicited by voltage ramps (40 to –100 mV) from CHO cells expressing wild type (A) and Gln573––Arg583 mutant (B) channels in the absence and presence of amiloride. As summarized in Fig. 13C, the amiloride-sensitive inward current at –80 mV of 0.16 ± 0.04 nA (n = 19) for channels containing the Gln573––Arg583 mutant was significantly less than the 2.3 ± 0.55 nA (n = 9) for wild type channels. In contrast to the large effect of deleting Gln573––Arg583, deleting Thr584––Pro600 had a very modest effect on Na+ transport as measured in FRT cells. The relative transport of

The summary graph in Fig. 12A compares Po for each channel with wt and mt channels having 0.67 ± 0.07 and 0.16 ± 0.03, respectively (n = 8). As mentioned above, patches made from CHO cells expressing wt channels most often contained several channels. Thus, Po for wt often had to be estimated from patches containing several channels when it was possible to reliably estimate n from all-point histograms. (Only 3 of the 11 patches containing wt channels contained one channel; however, only patches containing 5 channels or fewer were used to calculate Po.) This may have led us to overestimate Po in the wt group. Nevertheless, these results in conjunction with those in Figs. 5–8 and 11 strongly argue that channels containing the mt Gln573––Pro600 ENaC subunit have a decreased Po. The mean open and closed time data for wt (n = 3) and mt (n = 7) channels summarized in Fig. 12B reveals the possible mechanism resulting in decreased Po. The mean open time and mean closed time for wt channels were 344 ± 98 and 245 ± 105 ms compared with 63 ± 9 and 964 ± 147 ms for mt channels. Thus, mt channels spent significantly longer time in the closed state and significantly less time in the open state resulting in a decreased Po.

The functional domain following TM2 in γENaC was further defined by assessing the activity of channels containing Gln573––Arg583 and Thr584––Pro600 mutants. Fig. 13 shows representative currents elicited by voltage ramps (40 to –100 mV) from CHO cells expressing wild type (A) and Gln573––Arg583 mutant (B) channels in the absence and presence of amiloride. As summarized in Fig. 13C, the amiloride-sensitive inward current at –80 mV of 0.16 ± 0.04 nA (n = 19) for channels containing the Gln573––Arg583 mutant was significantly less than the 2.3 ± 0.55 nA (n = 9) for wild type channels. In contrast to the large effect of deleting Gln573––Arg583, deleting Thr584––Pro600 had a very modest effect on Na+ transport as measured in FRT cells. The relative transport of
1.0 ± 0.06 versus 0.86 ± 0.04 (p = 0.06, n = 18; not shown) for wt and mt channels, respectively, was not significantly different. These results are consistent with the idea that the 11 amino acids between Gln573–Arg583 contain the most significant residues with respect to support of ENaC activity.

DISCUSSION

The current study used a novel yeast one-hybrid screen to identify regions in the cytosolic tails of ENaC that localized to the plasma membrane complementing an inherent, temperature-sensitive growth defect in the Cdc25H yeast strain. Confocal images of CHO cells transfected with EGFP hybrids made from these cytosolic portions of ENaC and a membrane marker (ECFP-M) validated findings in yeast showing that the NH2-terminal tail of αENaC, but not β- and γENaC, and the COOH-terminal tails of all three subunits co-localized with the membrane marker. Together, these findings support the idea that these regions of ENaC are involved in protein-protein and/or protein-lipid interactions localized to or near the plasma membrane. The yeast screen was used to further localize membrane interacting domains within ENaC subunits. The functional significance of one membrane interacting region, residing within Gln573–Pro600 of γENaC, was accessed in the current study using a reconstituted mammalian expression system where ENaC was overexpressed in CHO cells and studied using patch clamp electrophysiology. This region has not been identified previously as playing a role in ENaC activity. We found that deletion of Gln573–Pro600 within the cytoplasmic COOH tail of γENaC decreased activity by affecting channel gating but not channel localization or oligomerization. The current results support the idea that the Gln573–Pro600 region of γENaC is not involved in channel oligomerization or targeting channels to the membrane but interacts with an as yet undefined membrane resident factor to support normal channel gating and activity. An alternative interpretation that cannot be excluded with the current data set is that, although the ΔGln573–Pro600 mt γENaC subunit interacted normally with α and β subunits and was localized to the plasma membrane, the mutant channel is inactive either because it is non-functional or in a prolonged quiescent state. The resulting macroscopic currents and single channel currents measured in mt transfectants then would have resulted from αβ heterodimeric or α homomeric channels. Nonetheless, this alternative also is consistent with the idea that the Gln573–Pro600 region in γENaC interacts with a membrane factor to facilitate formation of normal functional heterotrimetric channels.

Further deletion mutagenesis localized the active region in the COOH terminus of γENaC to reside within residues Gln573–Arg600. The 11 amino acids between Gln573–Arg600 contain several conserved regions: two tracts, KAK and RRR, containing positively charged residues and an absolutely conserved tryptophan repeat, WW. In consideration of the recent findings (48, 49) showing that ENaC activity is modulated by anionic phospholipids, which are well known to interact with positively charged residues, it is provocative to speculate that the conserved charged residues within this region of ENaC are somehow involved in this regulation. Interestingly, the general region just distal to TM2 in αENaC, including residues Met610–Val632, identified as local-
izing to the membrane in the current study has been identified previously by Volk et al. (19, 50) as playing an essential role in kinase regulation of the channel. This group showed that kinase regulation associated with this region was important for proper localization of ENaC to the plasma membrane. Specifically, these authors report that Pro595 of α-hENaC (correspond-
ing to Pro622 in α-ENaC) was necessary for normal channel activity and suppression of activity by the broad-spectrum kinase inhibitor staurosporine. Interpretations of these earlier data as presented by the authors included that Pro595 is involved in an interaction between ENaC and a staurosporine-sensitive kinase, a phosphoprotein, and alternatively, a linker protein that then binds a kinase or phosphoprotein. It is our contention that these earlier findings lend further support to the functional relevance of domains identified with the yeast one-hybrid screen in the present study. Notably, this region of ENaC, in addition, is well conserved across species and also within the ENaC subunit (51).

There are currently no reports that we are aware of specifically suggesting that the first 54 residues in the NH2 terminus of ENaC identified in the current study as membrane interactive are important for positive regulation of ENaC activity. However, Chalfant et al. (16) have identified a possible endocytic tag within this region corresponding to KGDK50 that may play a role in down-regulation of ENaC activity. It is our contention that these earlier findings lend further support to the functional relevance of domains identified with the yeast one-hybrid screen in the present study. Notably, this region of αENaC, in addition, is well conserved across species and also within the δENaC subunit (51).

There are currently no reports that we are aware of specifically suggesting that the first 54 residues in the NH2 terminus of αENaC identified in the current study as membrane interactive are important for positive regulation of ENaC activity. However, Chalfant et al. (16) have identified a possible endocytic tag within this region corresponding to KGDK50 that may play a role in down-regulation of ENaC activity. Moreover, we find the recent reports (28) provocative, showing that the NH2 terminus of α-ENaC is alternatively spliced and include alternative first exons to form unique αENaC subunits. Whereas in oocytes the alternative αENaC subunits do not show functional differences, it remains to be determined whether there is a tissue-specific activity difference between these apparently interchangeable subunits or whether these subunits respond in unique manners to different regulatory pathways. It is not clear at this time if there are similar NH2-terminal variant αENaC subunits in other species arising from differential splicing and inclusion of alternative exons; however, the early but not latter portions of the NH2-terminal tails of αENaC in general are heterogeneous across species. It is exciting to speculate that this apparently hyper-flexible region may possibly contribute to the noted species- and tissue-specific differences in channel activity.

Awayda et al. (12) demonstrated previously that injection of a 30-amino acid peptide with identity to the distal portion of the COOH terminus of the β subunit into Xenopus laevis oocytes expressing ENaC quickly decreased activity. From this and other results, it was concluded in this earlier study that this region of ENaC exerts tonic inhibition of channel activity with the implication being that this region of ENaC interacts with other regions of the channel to negatively regulate Po.A n alternative is that the exogenous peptide blocked activity by competing for a positive regulatory protein. Nonetheless, these previous findings provide support for the idea that the most distal portion of βENaC plays a functional role in control of channel activity. We demonstrate in the current study that the last 17 residues in βENaC are membrane-interactive, which is consistent with the possibility that this region plays some role in development of modulatory protein-protein and/or protein-lipid interactions. Consistent with this idea, Dinudom et al. (52) demonstrated that a peptide identical to the distal most 10 residues in βENaC inhibited ENaC activity in intralobular

**FIG. 12.** Channels containing the ΔGln573–Pro600 deletion mt have decreased Po.A A, summary graph comparing Po in wt and ΔGln573–Pro600 channels in outside-out patches. This is a single blinded population study sampling 3 distinct parallel transfections. *, p < 0.05 versus wt ENaC. B, summary graph comparing the mean open (MOT) and mean closed (MCT) times for wt and mt ENaC overexpressed in CHO cells. *, p < 0.05 versus wt.

**FIG. 13.** Channels containing the ΔGln573–Arg583 deletion mt have decreased activity. A and B, current from CHO cells expressing wild type (A) and the ΔGln573–Arg583 mutant (B) channel in the absence and presence of amiloride. Currents elicited by voltage ramps from 40 to −100 mV. Solutions are as above. C, summary graph comparing amiloride-sensitive inward current at −80 mV for wild type and mutant channels. *, significantly less versus wild type.
duct cells most likely by competing for a required positive regulatory protein.

We interpret the current results collectively as showing that specific residues/microdomains, of which Gln573 in γENaC had not been identified previously, play important roles in control of ENaC activity. The current results demonstrate that residues within Gln573–Arg583 interact with a membrane resident factor to support ENaC openings. We expect through technical assistance on EF microscopy experiments.

We thank Dr. Nikita Gamper for aid in reconstituting mENaC in CHO cells. We also thank Emily Adams for expert technical assistance on EF microscopy experiments. We thank Dr. Nikita Gamper for aid in reconstituting mENaC in CHO cells. We also thank Emily Adams for expert technical assistance on EF microscopy experiments.

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