Distinct Structural Elements in the First Membrane-spanning Segment of the Epithelial Sodium Channel*

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Epithelial Na+ channels (ENaCs) comprise three subunits that have been proposed to be arranged in either an α2βγ or a higher ordered configuration. Each subunit has two putative membrane-spanning segments (M1 and M2), intracellular amino and carboxyl termini, and a large extracellular loop. We have used the TOXCAT assay (a reporter assay for transmembrane segment homodimerization) to identify residues within the transmembrane segments of ENaC that may participate in important structural interactions within ENaC, with which we identified a candidate site within αM1. We performed site-directed mutagenesis at this site and found that, although the mutants reduced channel activity, ENaC protein expression at the plasma membrane was unaffected. To deduce the role of αM1 in the pore structure of ENaC, we performed tryptophan-scanning mutagenesis throughout αM1 (residues 110–130). We found that mutations within the amino-terminal part of αM1 had effects on activity and selectivity with a periodicity consistent with a helical structure but no effect on channel surface expression. We also observed that mutations within the carboxyl-terminal part of αM1 had effects on activity and selectivity but with no apparent periodicity. Additionally, these mutants reduced channel surface expression. Our data support a model in which the amino-terminal half of αM1 is α-helical and packs against structural element(s) that contribute to the ENaC pore. Furthermore, these data suggest that the carboxyl-terminal half of αM1 may be helical or assume a different conformation and may be involved in tertiary interactions essential to proper channel folding or assembly. Together, our data suggest that αM1 is divided into two distinct regions.

The epithelial Na+ channel (ENaC)3 is expressed in the apical membrane of sodium-absorptive epithelia that line the distal nephron, lung airway and alveoli, and descending colon and is the primary target of the potassium-sparing diuretic, amiloride. Expressed in these tissues, ENaC plays a critical role in maintaining Na+ homeostasis and controlling blood pressure and airway fluid volume. ENaC comprises homologous α, β, and γ subunits arranged in either a tetrameric αβγ or higher ordered arrangement (1–5). Each subunit has two membrane-spanning domains (termed M1 and M2), intracellular amino and carboxyl termini, and large extracellular domains. ENaCs are members of the Degenerin/ENaC family of cation-selective channels that have roles in mechanosensation and nociception.

The structure of the ENaC pore has been the subject of several studies. The pore has been shown to be composed of the M2 segments along with the residues immediately extracellular to these segments from all three subunits (6, 7). In the tetrameric αβγ model, the two α subunits are posited to be on opposite sides of the pore (2). Given that the pore-forming components of all three subunits are highly homologous (see Fig. 1), the pore is thought to have 4-fold pseudosymmetry such that all three subunits fold and contribute similarly to the pore. Previous studies showing that specific M2 residues are solvent-accessible are consistent with helical M2 segments that have one side facing the pore lumen (7, 8). Studies of M1 domains within members of the Degenerin/ENaC family are limited but suggest that some part of M1 may be solvent-accessible (8–10). A proposed model for the structure of the ENaC pore describes the pore as a double funnel, with the narrowest point at the selectivity filter and M2 lining a pore that widens toward the inner leaflet of the membrane (6, 11). In the absence of experimental evidence, M1 has been suggested to be helical and pack against M2 distal to the pore lumen by homology to the role of M1 in K+ channels (12).

In this study, we have used the TOXCAT assay to identify residues within the ENaC transmembrane domains that may participate in important structural interactions within the channel complex. This assay employs the cytoplasmic domain of ToxR to initiate transcription of a gene conferring chloramphenicol resistance to the host bacterium upon dimerization of this domain driven by a test transmembrane sequence. Using this assay, we identified a candidate site within αM1. We performed site-directed mutagenesis at this site and found that, although the mutants reduced channel activity, ENaC protein expression at the plasma membrane was unaffected. In an effort to deduce the role of αM1 in the pore structure of ENaC, we next performed tryptophan-scanning mutagenesis throughout

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*The abbreviations used are: ENaC, epithelial Na+ channel; M1, first membrane-spanning segment; M2, second membrane-spanning segment; N, number of channels; P0, open probability; MBS, modified Barth’s saline; BSA, bovine serum albumin; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide.

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Distinct Regions within ENaC αM1

αM1 (residues 110–130) with the intention of perturbing helix-helix interactions. Where endogenous tryptophans are found, we mutated them to alanine (Fig. 1). We found that mutations within the amino-terminal part of αM1 affected activity and selectivity with a periodicity consistent with this region having a helical structure. Changes in activity were not due to changes in surface expression but rather were caused by changes in the single channel properties of the mutant channels. In addition, we also observed a break in the putative helix structure so that the carboxyl-terminal half of αM1 had different properties than the amino-terminal half. These differences include no clear evidence of helicity and large effects on channel surface expression. Our data support a model in which the amino-terminal half of αM1 is α-helical and packs against structural element(s) that contribute to the ENaC pore. Our data also suggest that the carboxyl-terminal half of αM1 may be either helical or assume a different structure and may interact with structural elements distinct from those interacting with the amino-terminal part of αM1.

EXPERIMENTAL PROCEDURES

**TOXCAT Constructs**—Plasmid pccKAN was obtained from Dr. Donald Engelman (Biochemistry, Yale) and Bluescript plasmids encoding the human α, β, and γ subunits of the epithelial sodium channel from Dr. Anil Menon (Molecular Genetics, University of Cincinnati). pccKAN encodes the gene for the cholera toxin (ctx) promoter (13), as well as a truncated version of the toxR protein (toxR') lacking its membrane-spanning domain. pccKAN also includes unique Nhel and BamHI sites suitable for creating in-frame chimeric proteins having the general structure toxR'-test transmembrane segment-maltose-binding protein. To create chimeras in which the test transmembrane segment is derived from an epithelial sodium channel, primers were synthesized to amplify the coding sequences for the membrane-spanning segments of the human α, β, and γ subunits while simultaneously adding the required 5'- and 3'-flanking restriction endonuclease sites. Amplification products were gel-purified and cloned into pccKAN to yield the plasmids pKB52, -54, and -56. An analogous set of expression plasmids, selectable on kanamycin instead of ampicillin, was created in the pSU18 background to enable replication and maintenance of two plasmids in the same host. The sequences of all constructs were verified to guard against cloning artifacts.

**TOXCAT Random Mutagenesis and Screening**—The sequence encoding human ENaC αM1 was mutagenized using error-prone PCR. Primers were designed to anneal to regions of pKB52 immediately upstream of the Nhel site and downstream of the site for BamHI. Amplifications were done in the presence of 0.5–2 mM MnCl2 and with one pyrimidine nucleotide held at a limiting concentration (typically 10 μM). Following 30 cycles of amplification, PCR products were precipitated, cleaved with Nhel and BamHI, and cloned into pccKAN. Ligation reactions were transformed into *Escherichia coli* DH5α cells and then replica-plated to screen in parallel for growth on ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml). Typically, about half of the ampicillin-resistant colonies were able to grow on chloramphenicol. Colonies that displayed an ampicillin-resistant/chloramphenicol-sensitive phenotype were selected for sequence analysis of their membrane-spanning domain inserts.

~10% of the clones analyzed were found to contain single point mutations within this region, whereas the remaining sequences were dominated by frameshifts. >90% of the catR clones sequenced were wild type, but a small proportion contained single point mutations within the regions coding the membrane-spanning domains. Presumably, this latter set of mutants occurs at sites that are not essential for dimerization.

**Site-directed Mutagenesis and ENaC Expression**—All ENaC clones used for functional studies were mouse ENaC subunits whose cDNAs were inserted into pBluescript SK− (Stratagene, La Jolla, CA) (14). Point mutations were generated using a PCR-based method as previously described (15). Target mutations were confirmed by sequencing at the University of Pittsburgh DNA-sequencing facility. Capped cRNAs for mutant and wild-type mouse ENaC subunits were synthesized with T3 RNA polymerase (Ambion Inc., Austin, TX) from linearized DNA templates. Stage V and VI oocytes free of follicle cell layers were injected with 1–4 ng of cRNA for each mouse ENaC subunit/oocyte and incubated at 18 °C for 20–30 h in modified Barth’s saline (MBS) (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM HEPES, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamicin sulfate, pH 7.4).

**Two-electrode Voltage Clamp**—Two-electrode voltage clamp was performed using a DigiData 1320A interface and a GeneClamp 500B voltage clamp amplifier (Axon Instruments, Foster City, CA). Data acquisition and analyses were performed using pClamp, version 8.2, software (Axon Instruments) on a 1.5-GHz Pentium 4 PC (Gateway 2000 Inc., N. Sioux City, SD). Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) with a Micropipette Puller (Sutter Instrument Co., Novato, CA) and had a resistance of 0.5–5 megohms when filled with 3 M KCl and inserted into the bath solution. Oocytes were maintained in a recording chamber (Automate Scientific, San Francisco, CA) with 20 μl of bath solution and continuously perfused with bath solution at a flow rate of 4–5 ml/min. The bath solution contained either 110 mM NaCl or LiCl, and 2 mM KCl, 2 mM CaCl2, 10 mM HEPES, pH 7.4. In experiments using [2-(trimethylammonium) ethyl]methanethiosulfonate bromide (MTSET)
(Toronto Research Chemicals, North York, Ontario, Canada), the compound was dissolved in bath solution <1 min before application to the oocyte. Oocytes were continuously perfused in 1 mM MTSET for 4 min prior to measurements assessing ENaC activity. All experiments were performed at the ambient temperature (20–24°C).

**Measurements of ENaC Surface Expression—**Experiments were performed essentially as described previously (16). Oocytes were injected with 2 ng/subunit of αβγ or wild-type or mutant α subunits along with FLAG epitope-tagged β and wild-type γ subunits. Oocytes were blocked for 30 min in MBS supplement with 10 mg/ml bovine serum albumin (MBS/BSA) and then incubated for 1 h with MBS/BSA supplement with 1 μg/ml mouse monoclonal anti-FLAG antibody (M2) (Sigma) at 4°C. Oocytes were then washed at 4°C for 1 h in MBS/BSA and incubated with MBS/BSA supplemented with 1 μg/ml horse-radish peroxidase-coupled secondary antibody for 1 h at 4°C (peroxidase-conjugated AffiniPure F(ab’2) fragment goat antimouse IgG; Jackson ImmunoResearch, West Grove, PA). The cells were extensively washed (12 times over 2 h) at 4°C and transferred to MBS without BSA. Individual oocytes were placed in 100 μl of SuperSignal Elisa Femto maximum sensitivity substrate (Pierce) and incubated at room temperature for 1 min. Chemiluminescence was quantified in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

**Patch Clamp—**Vitelline membranes of oocytes were removed manually following incubation of the oocytes at room temperature in MBS supplemented with 200 mM sucrose. Oocytes were then transferred to a recording chamber with bath solution and allowed to recover for 10 min before clamping. The bath and pipette solutions contained 110 mM LiCl, 2 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.4. Glass pipettes with tip resistances of 5–20 megohms were used. For single channel measurements, single channel currents were recorded in an excised patch membrane, we employed a TOXCAT assay (13). In this experiment, the transmembrane and periplasmic domains of ToxR were assayed using recordings of duration ≥1 min with a Gaussian filter for display and analysis. The number of channels × open probability (NP) measurements were made using recordings of duration ≥5 min using Clampfit, version 9.2 (Axon Instruments).

**Statistical Analysis—**Data are presented as mean ± S.E., unless otherwise indicated. Statistical significance was analyzed by Student’s t test. Curve fittings were performed with Clampfit, version 9.0 (Axon Instruments Inc., Union City, CA) and Igor Pro, version 4.0.9.1 (Wavemetrics, Oswego, OR).

## RESULTS

**TOXCAT Screen of ENaC Transmembrane Segments—**To assess the ability of each of the human ENaC membrane-spanning segments to homodimerize within the context of a biological membrane, we employed a TOXCAT assay (13). In this assay, the transmembrane and periplasmic domains of ToxR are replaced by test transmembrane sequences and the mal-

tose-binding protein periplasmic domain, respectively. In such constructs, dimerization driven by putative membrane-spanning sequences subsequent to insertion into the membrane allows the cytoplasmic transcriptional activation domains (toxR) to interact with the ctx promoter, initiating transcription of a reporter gene encoding chloramphenicol acetyltransferase. The resulting chloramphenicol acetyltransferase activity can be measured by the resistance of bacterial cells hosting the plasmid to a chloramphenicol concentration gradient.

We created constructs suitable for the TOXCAT assay for each of the transmembrane segments (M1 and M2) for human ENaC subunits α, β, and γ. Upon transformation into DH5α cells, only the construct containing αM1 (pKB52) conferred chloramphenicol resistance up to 150 μg/ml. The remaining constructs did not confer chloramphenicol resistance, even at antibiotic concentrations as low as 10 μg/ml. This result indicates that human ENaC αM1 has the unique ability to drive homodimerization of the chimera when inserted into a bacterial inner membrane.

To determine which residues within αM1 were essential for the dimerization resulting in chloramphenicol resistance, we performed mutagenesis within αM1 using error-prone PCR and screened the resulting populations of mutants by TOXCAT (Fig. 2). Replica plating of ampicillin-resistant clones onto chloramphenicol enabled us to quickly identify a set of mutants that had been converted to a chloramphenicol-sensitive phenotype. As depicted in Fig. 2, mutations L90Q, L92H, L104H, and L105Q, each of which were obtained in our first mutagenesis experiment, all substantially reduced chloramphenicol resistance. L90Q was unable to grow in 25 μg/ml chloramphenicol, whereas the remainder were sensitive at 35–50 μg/ml. In a second experiment, these same mutants were obtained plus an L90R mutant that was chloramphenicol-resistant at 25 μg/ml.

We also analyzed 100 randomly selected clones that remained resistant to high chloramphenicol concentrations (125–150 μg/ml). The vast majority of these clones retained the wild-type sequence, but five contained mutations within the αM1 coding region, W91G, F95G, M97T, F100S, and L104P. We suggest that these positions are not important for the ability of αM1 to form dimers within the membrane, except for Leu-104. The mutants that weakened chloramphenicol resistance and therefore weakened the ability of αM1 to dimerize were L90Q, L92H, L104H, and L105Q. Of these, only L90Q was found in >1 clone. As both chloramphenicol-sensitive and -resistant mutants were isolated at Leu-104, we assumed that phenotypic changes were related to the specific nature of the substitution.

**Effect of Mutation at αLeu-115—**αL90Q had the strongest effect on αM1 dimerization, as measured by the TOXCAT assay. We therefore examined the effect of mutations at the cor-
Distinct Regions within ENaC αM1

responding site of mouse ENaC expressed in oocytes (αLeu-115) (Fig. 1). cRNAs encoding either wild-type α subunit, αL115A, or αL115F were co-injected with cRNAs encoding wild-type mouse β and γ subunits into Xenopus laevis oocytes. Twenty-four hours after injection, we measured amiloride-sensitive Na⁺ currents for the wild-type and mutant channels by two-electrode voltage clamp. We found that both mutations reduced ENaC-mediated Na⁺ currents so that the relative activities of αL115Aβγ and αL115Fβγ were 0.47 ± 0.06 (n = 21, p < 0.001) and 0.08 ± 0.02 (n = 12, p < 0.001), respectively.

To determine whether the reduction in Na⁺ current observed for each of these mutants was because of a reduction in the number of channels expressed at the cell surface or a change in the single channel properties of these mutants, we examined the surface expression for these two mutants. Surface expression was measured using a FLAG epitope-tagged β subunit (βF) and a chemiluminescence-based assay originally described by Zerangue et al. (16) and modified by Carattino et al. (17). The assay background measured with untagged αβγ-injected oocytes was 0.07 ± 0.02 relative light units/min. The surface expression of αβFγ (1.6 ± 0.2 relative light units/min) was similar to that of the αL115Aβγ (2.1 ± 0.2 relative light units/min, n = 12, p = not significant) and αL115FβFγ (1.6 ± 0.2 relative light units/min, n = 12, p = not significant) mutants.

Effect of M1 Tryptophan Mutations on ENaC Activity—The above data suggest that mutations in αM1 that disrupt αM1 dimerization, as measured by TOXCAT, reduce channel activity but do not significantly reduce channel surface expression. We hypothesized that αM1 is an α-helix (as has previously been predicted (18, 19)), which presents a face onto which another helix may bind. In the TOXCAT assay, the second helix must be αM1, but in fully assembled ENaC, the second helix may be αM2 or either M1 or M2 from the β or γ subunits. In this context, we note that when β or γ M1 constructs were co-transformed with pKB52 (αM1), there was a small but reproducible reduction in chloramphenicol resistance. To define and probe the interaction surface, we performed scanning tryptophan mutagenesis throughout αM1 (residues 110–130). Where tryptophans naturally occurred (αTrp-112, -116, and -125) we have instead mutated these residues to alanine to maximize the change in volume. Both tryptophan and alanine are well tolerated in α-helices (20). Because tryptophan has a large bulky hydrophobic side chain, it should be better accommodated at positions that interact with lipid than at those involved in interactions with other residues within the protein. Likewise, the large loss in residue volume resulting from tryptophan → alanine substitution should be better accommodated at positions that interact with lipid than positions that interact with other residues. If αM1 is α-helical, then positions that interact with other residues, or positions that interact with lipid for that matter, should have a periodicity near 3.6.

As above, cRNAs encoding wild-type or mutant α subunits of ENaC were mixed with those encoding wild-type β and γ subunits and injected into X. laevis oocytes. After 20–30 h, amiloride-sensitive Na⁺ currents were determined by perfusing expressing oocytes with a buffer containing 110 mM NaCl in the absence and presence of 100 μM amiloride and measuring the resulting currents by the two-electrode voltage clamp technique (Fig. 3A). All currents were normalized to the current measured for wild-type ENaC on the same day. The effect of mutation at each position varied from a 70% increase to a 98% decrease in Na⁺ currents, relative to wild-type ENaC (Fig. 1). For mutations at residues 110–121, there was a marked periodicity in the relative Na⁺ currents, such that mutations at positions 110, 113, 117, and 120 had similar or larger Na⁺ currents compared with wild-type ENaC, whereas mutations at 112, 115, and 119 had <10% of the wild-type activity of ENaC. To measure the periodicity, we fit this data to a simple sine function (Equation 1),

\[
y = A \cdot \sin \left( \frac{2\pi x}{\nu} + \phi \right) + y_0 \quad \text{(Eq. 1)}
\]

where \( A \) is a scaling factor, \( x \) is the residue number, \( \phi \) and \( y_0 \) are horizontal and vertical offsets, respectively, and \( \nu \) is the periodicity. The best fit value for \( \nu \) for mutations at residues 110–121 was 3.4 ± 0.1, similar to a predicted periodicity of 3.6 for an α-helix. Omitting tryptophan→alanine mutants αW112A and αW116A (Fig. 3A, filled circles) from the analysis yielded a similar fit with an identical \( \nu \) value (fit not shown). For mutations at residues 122–130, little periodicity remained evident, although the effect of mutation at residues 124–129 on relative Na⁺ currents were to moderately lower the activity (25–70%).

We also determined the effect of αM1 tryptophan mutations on ion selectivity (Fig. 3B). To determine the selectivity of these mutants for K⁺ and Li⁺, we perfused oocytes injected with each mutant with buffers containing 110 mM KCl or 110 LiCl in the absence and presence of 100 μM amiloride and measured the resulting currents by the two-electrode voltage clamp technique. Although there was no measurable K⁺ permeation (data not shown), all channels had measurable Li⁺ currents. However Li⁺ and Na⁺ currents for αL130Wβγ were too low to reliably determine a value for Li⁺:Na⁺ selectivity. Compared with wild-type ENaC, whose Li⁺:Na⁺ selectivity was 1.75 ± 0.07, the Li⁺:Na⁺ selectivity of the αM1 mutants varied from 1.1 ± 0.2 to 3.4 ± 0.6. Interestingly, for residues 110–121, Li⁺:Na⁺ selectivity correlated inversely with both Na⁺ and Li⁺ currents, giving \( r^2 \) values of 0.92 and 0.89, respectively (Fig. 3C). As such, a similar periodicity was observed for the Li⁺:Na⁺ selectivity values for residues 110–121 as was described above for relative Na⁺ currents (fit not shown). In contrast, for residues 122–129 there was no correlation between Li⁺:Na⁺ selectivity and either relative Na⁺ currents (\( r^2 = 0.47 \)) or relative Li⁺ currents (\( r^2 = 0.28 \)).

Effect of αM1 Tryptophan Mutations on ENaC Surface Expression—To determine whether the changes in Na⁺ current observed for the αM1 tryptophan mutants were attributable to changes in the number of channels expressed at the cell surface, we measured the surface expression of selected αM1 tryptophan mutants using a β subunit with an external epitope (FLAG) tag. The selected mutants ranged from largely inactive (αT119W, αL130W) to more active than wild type (αF120W), with four additional mutants between both extremes (αG121W, αA113W, αQ126W, and αA128W). The four mutants between residues 110 and 121 had surface expression
similar to wild-type (αβγ) ENaC (Fig. 4). However, the three mutants between 122 and 130 had reduced surface expression compared with wild-type ENaC. In fact, these three mutants had values for relative surface expression that were similar to their values for relative Na\(^+\) currents. These data complement the activity and selectivity data for these mutants in that they suggest that αM1 comprises two distinct regions.

**Effect of αG121W Mutation on ENaC Single Channel Properties**—We observed that the αG121W mutant reduced Na\(^+\) currents and increased Li\(^+\)/Na\(^+\) selectivity compared with wild-type ENaC but was expressed similarly to wild-type ENaC on the surface of Xenopus oocytes. We hypothesized that the functional effects of mutation must therefore be due to changes in the single channel properties of the mutant channel. Thus, we performed single channel recordings of the αG121W mutant channel (Fig. 5). In excised inside-out patches in which both the bath and the pipette contained identical solutions and Li\(^+\) was the charge carrier, the single channel conductance was 8.7 ± 0.1 picosiemens, compared with a reported value of 8.1 picosiemens for wild-type ENaC (21). From these recordings, we also estimated the N\(P_o\) to be 0.03 ± 0.02, which is lower than the \(P_o\) value of ~0.5 (but may vary from 0.1–0.8) reported for wild-type ENaC (22, 23). These data suggest that a reduced \(P_o\) value largely accounts for the low activity of the αG121W mutant.

**Accessibility of αM1 Residues to Extracellular Solution**—Poët et al. (9) observed that cysteines engineered at several sites within the M1 segment of FaNaC, an ENaC/Deg family member, are accessible to externally applied MTSET. To assess the relevance of these data to ENaC structure, we generated single cysteine mutations at α subunit residues 114–121 (there is an endogenous cysteine at 118) and measured their ability to confer externally applied MTSET sensitivity to the mutant channels. These sites were chosen due to their presence in the part of M1 displaying clear periodicity with the tryptophan mutations (see above), their overlap of some of the analogous MTSET-sensitive sites in the Poët et al. paper (9), and to sample two full turns of the putative αM1 helix.

We employed the α5583C mutant as a positive control (24). Although MTSET inhibited the α5583C mutant (75 ± 4% inhibition), none of the other mutants were MTSET-sensitive (−12 to 7% inhibition). These data suggest that these sites are not accessible to externally applied MTSET.

**DISCUSSION**

Previous studies have suggested that the M2 transmembrane domains from each ENaC subunit are helical and form the pore with their acidic residues pointing toward the pore lumen (7, 8). In contrast to M2, experimental evidence for the role of M1 in the structure of the ENaC pore is limited. In an M1-substituted cysteine accessibility study on the Phe-Met-Arg-Phe-amide-activated Na\(^+\) channel (FaNaC), a channel that shares ~17% sequence identity with ENaC subunits, Poët et al. (9) concluded that the M1 domains of FaNaC line the channel pore, because cysteine residues introduced at seven different positions in M1 were accessible to modification by externally applied sulfhydryl reagents. In a substituted cysteine accessibility study on an acid-sensing ion channel (ASIC 1a), a channel that shares 18–20% sequence identity with ENaC subunits, Pfister et al.
(10) identified one residue at the amino-terminal end of M1 that was accessible to modification to internally or externally applied Cd²⁺. Based on homology to K⁺ channels (25) and predictive algorithms, others have suggested that M1 is helical and may interact with pore-lining M2 within the same subunit (12).

Our data support a helical model for the amino-terminal portion of αM1. Both the relative activity and ion selectivity data varied periodically as residues 110–121 were systematically mutated (Fig. 3, A and B). The calculated periodicity was 3.4 ± 0.1, which is consistent with the predicted periodicity of 3.6 for an α-helix. However, the observed periodicity largely disappears when residues carboxyl-terminal to αGly-121 are mutated. These results are complementary to surface expression data indicating that mutations at or before αGly-121 do not affect surface expression, whereas mutations at residues beyond position 121 reduce surface expression concomitant with the relative Na⁺ current of that mutant. Together, they indicate that αM1 is organized into two distinct regions (Fig. 6, B and C). The amino-terminal region is likely α-helical, having one face of its helix binding another transmembrane segment. The carboxyl-terminal region may also be α-helical, based on secondary structural predictions, or it may have a different secondary structure (Fig. 6A). In the case that the carboxyl-terminal region is α-helical, there may be a break in the αM1 helix akin to the break in the helix of the S6 segment of voltage-dependent K⁺ channels (26, 27). Mutations in the carboxyl-terminal region that led to reduced surface expression suggest that those mutants cause defects in either channel folding or assembly.

Our experiments do not fully address the issue of solvent accessibility. None of the α114–α121 cysteine mutants were significantly affected by externally applied MTSET. These data suggest that either these residues do not face the pore or these residues face the pore but are not accessible to externally applied MTSET. Thus our data for ENaC contrast with data previously observed for FaNaC. This may be due to a more restrictive ENaC selectivity filter, structural differences in the position of M1, or a combination of both factors. We did not apply MTSET to the intracellular...
FIGURE 6. Proposed models for role of αM1 in ENaC pore structure. A, predicted secondary structure for αM1 using GOR-V (31), nnPredict (32), GARNIER (33), and other algorithms. Other algorithms included JPRED (34), PSIPRED (35), and Chou-Fasman (20). B and C, these models depict αM1 as being divided into two regions, with the transition between the amino- and carboxyl-terminal regions occurring near αGly-121. Only the α subunit is shown for clarity. In both models, a subunit residues 112, 115, and 119 lie within a helix-helix interface, where the interacting helix may be αM2. We propose that the carboxyl-terminal region may be helical (β) or assume a more extended structure (C) and may interact with the selectivity filter and/or neighboring subunits. Not depicted, αM1 may also lie in a position to allow for solvent accessibility, as has been proposed for other members of the ENaC/Deg gene family (9, 10).

lar side of ENaC because of the large number of cysteine residues that were observed to confer sensitivity to intracellularly applied MTSET (28).

Although our experiments do not fully address the issue of solvent accessibility of αM1 residues, as discussed below, our data are more consistent with an ENaC pore model in which αM1 interacts with a pore-lining component of ENaC rather than lining the pore itself. The TOXCAT assay measures the ability of a test sequence to homodimerize. The residues identified to be important in human αM1 dimerization were (in mouse numbering) α-Leu-115, α-Leu-117, α-Leu-129, and α-Leu-130, whereas those whose mutation did not affect αM1 dimerization were αTrp-116, αPhe-120, αMet-122, and αPhe-125. Despite the fact that the TOXCAT assay measures something quite different from the effects of mutation on channel activity, there is remarkable agreement between these two sets of data. For those sites where mutations largely eliminated channel activity, mutations also prevented αM1 dimerization (α-Leu-115 and -130). For those sites where mutations increased channel activity, mutation did not affect αM1 dimerization (αPhe-120 and αMet-122). For those sites where mutations did not alter or moderately decreased channel activity, the effect of mutations on dimerization varied. Mutations at αTrp-116 and αPhe-125 did not affect dimerization, whereas mutations at α-Leu-117 and -129 did. Although some of these latter differences can be attributed to the different nature of each experiment, much of this difference may be attributed to the specific mutant that was tested in the TOXCAT experiments. This point is illustrated by the ability of αL129H to prevent αM1 dimerization, whereas αL129P had no effect. Thus, we suggest that the residues where mutations largely eliminated channel activity are likely involved in interactions with residues on other membrane-spanning segments within ENaC.

The Li⁺:Na⁺ selectivity data for the αM1 tryptophan mutants also provide support for a model in which αM1 interacts with a pore-lining component of ENaC. For the aminoterminal part of αM1, we found that the effects of mutations on ion selectivity correlated with effects on whole cell currents, such that the mutants that had low Na⁺ or Li⁺ currents had higher Li⁺:Na⁺ selectivity ratios (Fig. 3C). Thus both effects are dependent on residue position. Using the model in which αM1 interacts with a pore-lining structure leads to the interpretation that introducing (or removing) bulky tryptophans at this interface alters the positions of the pore-lining structural elements and pore geometry. Introduction (or removal) of tryptophans that interact with lipid are likely to have little or no effect on pore structure. Changes in pore geometry could result in differential effects on Li⁺ and Na⁺ conductivity and could also alter channel gating if the affected structural elements had a dynamic role in gating. Indeed, single channel recordings of the αG121W mutant showed a small increase in Li⁺ conductivity and a large decrease in channel P₀. This decrease in channel P₀ may be analogous to the effect of mutation at the break in the S6 helix of voltage-dependent K⁺ and Na⁺ channels (29, 30). Alternatively, if αM1 lined the pore, the introduction of bulky tryptophans into the pore might alter Li⁺:Na⁺ selectivity. However, in such a model, it is unclear why the mutants αW112A, αL115W, and αT119W would have similar effects. Because αM1 is helical between residues 110 and 121, if αM1 lined the pore, the functional groups of one face of the helix would be accessible to permeating ions. If these three sites faced the pore, we would predict some correlation between the identity of the mutated functional group and the functional effect of the mutation, which we did not observe.

Thus, it seems likely that sites at which mutation to tryptophan largely eliminated channel activity and altered channel selectivity are also sites where αM1 interacts with other ENaC structural elements. In the TOXCAT experiments, the other element was αM1 itself. Current models of the ENaC pore, based on a tetrameric (αβγ) stoichiometry, posit an αβαγ arrangement around the pore of the channel (2). Based on M2 functional data and analogy to K⁺ channels, the M2 segments of each subunit line the pore and the M1 segment of each subunit may pack against the M2 segment from the same subunit (7, 8). Such a model would exclude αM1-αM1 interactions. One modification of this model to allow αM1-αM1 interactions would have αM1 line the pore in at least the closed state at specific locations (α-Leu-115/117 and/or α-Leu-129/130). Another modification of this model to allow αM1-αM1 interactions would be a double-barreled (αβγγγ) ENaC channel, where the two barrels are bound at an αM1-αM1 interface. Such a model would be consistent with recent studies supporting a minimum of two β and two γ subunits per ENaC oligomer (4, 5). However, there is no evidence to support a double-barreled ENaC channel.
Distinct Regions within ENaC αM1

We propose that the binding face that αM1 presents, which allows for αM1 homodimerization, is also favorable for binding αM2 in an anti-parallel fashion, as current models propose. Alternatively, αM1 might instead bind the M1 or M2 segments from either the β or γ subunits. If αM1 was bound to either the β or γ subunit, we would predict that disruption of that interface would disrupt channel assembly, and therefore cell surface expression should decrease. In fact, we observed that mutations in the carboxyl-terminal part of αM1 decreased surface expression. We also observed that co-transformation of β or γ M1 constructs with the αM1 construct in the TOXCAT assay slightly reduced chloramphenicol resistance. These observations suggest that the carboxyl-terminal part of αM1 has an important role in channel assembly. If the carboxyl-terminal part of αM1 interacted with residues within and flanking the selectivity filter (which is narrow), αM1 could be in a position to facilitate channel assembly. Such a model could account for how mutations in this region affect activity, selectivity, and surface expression without directly contributing to the pore. For mutations in the amino-terminal part of αM1, we observed no effect on surface expression. We also found no correlation between the type of the amino acid being mutated to tryptophan and the effect on channel activity. Rather, the position within the amino-terminal part of αM1 was the sole determining factor in whether mutation to tryptophan increased or decreased channel activity. Furthermore, changes in ion selectivity also correlated with residue position rather than with the type of the amino acid being mutated to tryptophan. These data are consistent with a model in which this region of αM1 interacts with αM2, such that mutations in αM1 that alter channel activity and selectivity do so indirectly by changing the residue volume within the αM1-αM2 interface and ultimately pore diameter or pore-lining residue interaction. In this model, we have interpreted changes in channel activity due to mutations to be directly related to the extent of contact between αM1 and αM2.

In summary, our results suggest that αM1 is organized into two distinct regions, with the transition between regions occurring near αGly-121. The amino-terminal region is characterized by an α-helical secondary structure where mutations on one face of the helix decreased channel activity and increased Li⁺:Na⁺ selectivity without affecting channel delivery to the membrane surface. The carboxyl-terminal region may be helical or may have a different secondary structure, but Trp mutations there generally decreased channel activity without a correlated effect on Li⁺:Na⁺ selectivity. That these latter mutations also decreased channel surface expression suggests that mutations there disrupt channel assembly or folding. Finally, αM1 was found to dimerize in the TOXCAT assay. Mutations at sites identified to be at the dimerization interface also decreased channel activity, suggesting that these sites lie within an interface between structural elements within ENaC.

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