Second Transmembrane Domains of ENaC Subunits Contribute to Ion Permeation and Selectivity*

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Shaohu Sheng‡‡, Kathleen A. McNulty††, Johanna M. Harvey‡‡, and Thomas R. Kleyman‡‡‡

From the Departments of Medicine and of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261 and the Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Epithelial sodium channels (ENaC) are composed of three structurally related subunits (α, β, and γ). Each subunit has two transmembrane domains termed M1 and M2, and residues conferring cation selectivity have been shown to reside in a pore region immediately preceding the M2 domains of the three subunits. Negatively charged residues are interspersed within the M2 domains, and substitution of individual acidic residues within human α-ENaC with arginine essentially eliminated channel activity in oocytes, suggesting that these residues have a role in ion permeation. We examined the roles of M2 residues in contributing to the permeation pore by individually mutating residues within the M2 domain of mouse αENaC to cysteine and systematically characterizing functional properties of mutant channels expressed in Xenopus oocytes by two-electrode voltage clamp. The introduction of cysteine residues at selected sites, including negatively charged residues (Glu595, Glu598, and Asp602), led to a significant reduction of expressed amiloride-sensitive Na+ currents. Two mutations (E595C and D602C) resulted in K+-permeable channels whereas multiple mutations altered Li+/Na+ current ratios. Channels containing D602K or D602A also conducted K+ whereas more conservative mutations (D602E and D602N) retained wild type selectivity. Cysteine substitution at the site equivalent to Asp602 within β mENaC (βD544C) did not alter either Li+ or K+/Na+ current ratios, although mutation of the equivalent site within γ mENaC (γD562C) significantly increased the Li+/Na+ current ratio. Mutants containing introduced cysteine residues at Glu595, Glu598, Asp596, or Thr607 did not respond to externally applied sulfhydryl reagent with significant changes in macroscopic currents. Our results suggest that some residues within the M2 domain of αENaC contribute to the channel’s conduction pore and that, in addition to the pore region, selected sites within M2 (Glu595 and Asp602) may have a role in conferring ion selectivity.

The epithelial sodium channel (ENaC) mediates Na+ transport across high resistance epithelia and has a key role in Na+ homeostasis and blood pressure control. This channel is a member of the ENaC/degenerin gene superfamily and is composed of three structurally related subunits, termed α, β, and γ (1). Members of the ENaC/degenerin family are homo- or hetero-oligomeric proteins whose subunits share a common topology of two membrane spanning domains (M1 and M2) and intracellular amino and carboxyl termini. All three ENaC subunits contribute to the formation of the ion-conducting pore (2–7).

Hydropathy analyses of ENaC subunits have identified two large hydrophobic domains consisting of about 45 residues. The second hydrophobic domain contains two regions that are distinct in structure and function. The amino-terminal region is functionally similar to the pore region of many cation channels and is a key element determining pore properties of ENaC, including selectivity, gating, conductance, and the binding of channel blockers (2–8). On the other hand, the carboxyl-terminal regions of the second hydrophobic domains of ENaC subunits are predicted to have an α helical structure, similar to the sixth transmembrane domains (S6) of voltage-gated K+, Na+, and Ca2+ channels and to the second transmembrane domains of inward rectifier K+ channels.

Recent work suggests that M2 domains of ENaC also contribute to the formation of the pore. Langloh et al. (9) reported that mutations of the negative-charged amino acids within the M2 domains of human αENaC (E568R, E571R, and D575R) nearly eliminated channel activity, although these mutations did not alter the levels of protein expression at the plasma membrane. Furthermore, mutations of positively charged residues immediately following the α-subunit M2 domain altered cation selectivity of human ENaC (10). To explore the functional roles of residues within the M2 domains of ENaC, 18 residues (αVal593, αMet510) within M2 of the α subunit of mouse ENaC (mENaC) were individually mutated to cysteine. These mutant α-subunits were co-expressed with wild type β and γ mENaC subunits in Xenopus oocytes and analyzed using the two-electrode voltage-clamp technique. Our results suggest that M2 residues participate in formation of the ENaC pore and that several negatively charged residues may have a role in restricting K+ permeation through the pore.

MATERIALS AND METHODS

Site-directed Mutagenesis and Functional Expression—Single point mutations within M2 domains of mouse α, β, or γ ENaC subunits were generated by polymerase chain reaction as previously described (6). Target mutations were confirmed by direct DNA sequencing at the University of Pennsylvania DNA sequencing facility. Capped complementary RNAs (cRNAs) for mutant and wild type mENaC subunits were synthesized with T3 RNA polymerase (Ambion Inc., Austin, TX)

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†† Recipient of a postdoctoral fellowship award from the Cystic Fibrosis Foundation.

‡‡ To whom correspondence should be addressed: Renal-Electrolyte Division, A919 Scule Hall, 3550 Terrace St., Pittsburgh, PA 15261. Tel.: 412-647-3121; Fax: 412-648-9166; E-mail: kleyman@pitt.edu.

† The abbreviations used are: ENaC, epithelial sodium channel; mENaC, mouse ENaC; hENaC, human ENaC; rENaC; rat ENaC; M2, second transmembrane domain; S6, the sixth transmembrane domain; K+, inward rectifier K+ channel; KcsA, K+ channel from Streptomyces lividus; cRNA, complementary RNA; MTSET, [(2-trimethylammoni- methyl)ethyl] methanethiosulfonate bromide.

Kir, inward rectifier K+ channel; KcsA, K+ channel from Streptomyces lividus; cRNA, complementary RNA; MTSET, [(2-trimethylammoni- methyl)ethyl] methanethiosulfonate bromide.

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Fig. 1. Sequence alignments, secondary structural predictions, and helical wheel analyses of mENaC M2 domains. A, amino acid sequence alignments of the M2 domains of α, β, γ mENaC (GenBank accession numbers AF112185, AF112186, and AF112187), δENaC (δ subunit of human ENaC, U38254), bENaC1 (human brain sodium channel, Q16515), ASIC1 (acid-sensing ion channel 1, or proton-gated cation channel 1, P55926), NaChBفر (FMRFamide-activated amiloride-sensitive sodium channel, Q20511), DEG-1 (degenerin from Caenorhabditis elegans, P24585), and MEC-4 (mechanosensitive protein from C. elegans, U53669). The multiple sequence alignment was performed with MacVector version 6.5 (MacVector) on a PowerPC (Apple). Identical amino acids are shaded, and similar residues are boxed. Residues Val355, Met350 from omENaC that were mutated in the present study are shown on the top line. B–D, secondary structure predictions of M2 domains in α, β, and γ mENaC, respectively. Secondary structure predictions were performed with DNAStr 2.6 for Windows (Hitachi Software Engineering Co., Ltd., South San Francisco, CA) using the Chou-Fasman algorithm. The predicted secondary structure for each residue is displayed in the left panel. Uppercase letters indicate a high probability, and lowercase letters indicate a possibility that the residue occurs in the indicated conformation. Numbers in parentheses indicate the sequence number of the first residue in the sequence. Underlined residues preceding M2 domains have been identified as key sites forming a selectivity filter. The right panel shows the results of helical wheel analyses of M2 domains of α (B), β (C), and γ (D) mENaC. Amino acid residues are shown in three-letter code, and polar residues are in boldface.

from linearized DNA templates. Stage V and VI Xenopus laevis oocytes were injected with 2–4 ng of cRNA for each subunit in 50 nl of H2O. Injected oocytes were maintained at 18 °C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM Hepes, 0.3 mM CaCl2, 0.82 mM MgSO4, 10 µg/ml sodium penicillin, 10 µg/ml streptomycin sulfate, 100 µg/ml gentamicin sulfate, pH 7.4).

two-electrode Voltage Clamp—Two-electrode voltage clamp was performed at room temperature (20–24 °C) 24–72 h following cRNA injection. Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) and filled with 3 M KCl. Pipettes with tip resistance of 0.5–3 MΩ were chosen for experiments. Three bath solutions were used to determine the selectivity of the wild type or mutant mENaCs. The solutions contained 110 mM NaCl or LiCl or KCl, 2 mM CaCl2, and 10 mM Hepes, pH 7.4, adjusted with NaOH, LiOH, or KOH, respectively. For each bath solution total macroscopic currents and remaining currents in the presence of 100 µM amiloride were measured at the clamping voltage of −100 mV. Amiloride-sensitive currents were calculated by subtracting the latter from the total currents. Cation selectivity is presented as the ratio of amiloride-sensitive current in K+ or Li+ bath solution relative to the amiloride-sensitive Na+ current (Ic/IsNa and Ic/IsK).

Accessibility of external sulfhydryl reagent to mENaCs was examined as previously described (7). Briefly, 1 mM [2-(trimethylammoni um)ethyl] methanethiosulfonate bromide (MTSET) was prepared in the bath solution freshly and delivered to the recording chamber at the flow rate of 5–6 mℓ/min. Ratios of amiloride-sensitive Na+ currents recorded at −100 mV at 2 min after starting perfusion of MTSET and before perfusion were used to define the effects of this reagent on wild type and mutant channels.

Statistical Analysis—Data are presented as mean ± S.E. unless otherwise stated. Student’s t test was performed to statistically compare the differences between wild type and mutant channels using Microsoft Excel 97.

RESULTS

Our previous analyses of the secondary structure of the pore region of omENaC suggested that residues Ser592–Leu612 (or up to Phe615) form the a-helical membrane-spanning domain (6). Although the three ENaC subunits share only limited overall sequence homology at amino acid level (33–37%), the M2 domains within the three ENaC subunits share greater than 50% sequence similarity (Fig. 1A). This region is also highly conserved within other members of the ENaC/degenerin family. Polar residues are interspersed throughout the M2 domains of α, β, and γ mENaC and are predicted to line one face of the α helix (Fig. 1, B, C, and D).
Mutations within mENaC M2 Domains Result in Reduced Amiloride-sensitive Na⁺ Currents—All αβγ mENaCs containing substituted cysteine residues within the M2 domain of αmENaC expressed amiloride-sensitive Na⁺ currents, although levels of current expression varied (Fig. 2A). Interestingly, all mutants with cysteine substitutions of negatively charged residues displayed smaller currents than wild type, consistent with the observations of Langloh et al. (9). Because levels of Na⁺ current expression vary between different batches of oocytes, we compared current expression of wild type and mutant mENaCs in a paired manner. Wild type or mutant αmENaC together with wild type β and γ mENaC cRNAs were co-injected into oocytes obtained from a single batch of oocytes. Amiloride-sensitive whole cell Na⁺ currents were determined in oocytes expressing wild type αβγ mENaC, αE595Cβγ, αE598Cβγ, αD602Cβγ, or αT607Cβγ. As shown in Fig. 2B, expression of αE595Cβγ, αE598Cβγ, or αD602Cβγ led to a significantly reduced amiloride-sensitive whole cell Na⁺ currents when compared with wild type. Furthermore, substitution of αAsp⁶⁰² with E, K, or N, or substitution of a cysteine residue at the analogous sites within β (βAsp⁵⁴⁴) or γ (γAsp⁵⁶⁵) mENaC led to a significant reduction in amiloride-sensitive whole cell Na⁺ currents (Fig. 2C). The reduction of expressed whole cell Na⁺ currents observed with channels containing conservative substitutions of αAsp⁶⁰² (αD602E, αD602N) was modest, when compared with channels with non-conserved substitutions (αD602C and αD602K). No detectable amiloride-sensitive Na⁺ currents were observed in oocytes injected with double (αβD544CγD562C) or triple mutants (αD602CβD544CγD562C). Although the exact role of the negatively charged residues within M2 domains of ENaC subunits is not clear, these results suggest that polar residues in M2 domains have an important role in the functional expression of ENaC, as suggested by Langloh et al. (9).

Selected Mutations within ENaC M2 Domains Altered Cation Selectivity—M2 domains likely form part of the ENaC conduction pore in a manner similar to S6 of voltage-gated cation channels and M2 of inward rectifier K⁺ channels. Furthermore, residues within M2 may have a role in maintaining the cation-selective characteristics of ENaC. We examined whether mutations within the M2 domain of αmENaC result in alterations in the cation-selective phenotype by determining the amiloride-sensitive Li⁺/Na⁺ and K⁺/Na⁺ current ratios. Wild type channels have an I<sub>Li</sub>/I<sub>Na</sub> of 1.98 ± 0.06 (n = 12), and an I<sub>K</sub>/I<sub>Na</sub> of −0.03 ± 0.01 (n = 12). Two mutant channels, αE595Cβγ and αD602Cβγ, exhibited measurable inward amiloride-sensitive K⁺ currents at the clamping voltage of −100 mV with K⁺/Na⁺ current ratios of 0.11 ± 0.02 (n = 12).
and 0.12 ± 0.06 (n = 8), respectively. The $I_{K}/I_{Na}$ of αM610Cβγ was statistically higher than that of wild type (0.02 ± 0.01, n = 7, $p < 0.01$), however, the mutant channel was still highly selective for Na$^+$ over K$^+$ (50:1). αMet$^{109}$ of mENaC is aligned to αMet$^{283}$ of human ENaC that is located in proximity to residues αArg$^{286}$-Arg$^{287}$ (Fig. 6B). It has been shown that αR586E-R587E significantly increased K$^+$ and Li$^+$ permeabilities relative to Na$^+$ (10). Several mutations (αV593C, αV594C, αM596C, αA597C, αE595C, αE598C, αE1001C, αD602C, αL603C, αL604C, and αT607C) led to a moderate increase in $I_{K}/I_{Na}$, whereas αE595C resulted in a moderate decrease in $I_{K}/I_{Na}$ (Fig. 3).

The role of αAsp$^{602}$ in contributing to the cation selectivity of ENaC was further examined by introducing several different amino acid residues at this site. αD602Kβγ and αD602Aβγ exhibited K$^+$/Na$^+$ current ratios that were significantly greater than wild type. In contrast, conservative mutations (αD602N and αD602E) did not alter $I_{K}/I_{Na}$ (Fig. 4). Moreover, mutations at the sites corresponding to αAsp$^{602}$ within β or γ mENaC (βD544C or γD562C) did not alter $I_{K}/I_{Na}$. Although αD602C and γD562C led to small but significant increases in $I_{K}/I_{Na}$, the other mutants (αD602K, αD602A, αD602N, αD602E, and βD544C) displayed Li$^+$/Na$^+$ current ratios (measured at −100 mV) similar to that of wild type (Figs. 4). Representative voltage clamp recordings and current-voltage curves for wild type and selected mutant mENaCs are shown in Fig. 5. As shown in

![Figure 3](image-url)  
**Fig. 3.** Cation selectivity of wild type and mutant mENaCs. A, ratios of K$^+$ and Na$^+$ currents. Oocytes were injected with αβγ mENaC cRNAs or α mutant together with wild type βγ mENaC cRNAs. Inward currents were measured at −100 mV from oocytes bathed in Na$^+$ or K$^+$ bath solution. The ratios of $I_{K}/I_{Na}$ were calculated from amiloride-sensitive currents measured in the presence of a K$^+$ or Na$^+$ bath solution. B, ratios of Li$^+$ and Na$^+$ currents. $I_{K}/I_{Na}$ was determined from amiloride-sensitive currents measured at −100 mV in the presence of a Li$^+$ or Na$^+$ bath solution. Data are presented as mean ± S.E. from 13 oocytes for wild type and 6–14 oocytes for mutants. Open bars represent current ratios of wild type or mutant channels whose values were not significantly different from that of wild type. Filled bars indicate that the current ratios of mutant channels were statistically different from that of wild type at the significance level of 0.01 for $I_{K}/I_{Na}$ or 0.05 for $I_{L}/I_{Na}$.

![Figure 4](image-url)  
**Fig. 4.** Effects of mutations at αAsp$^{602}$, βAsp$^{544}$, and γAsp$^{562}$ on cation selectivity. Bars represent ratios of amiloride-sensitive K$^+$ (A) or Li$^+$ (B) currents relative to amiloride-sensitive Na$^+$ currents. Filled bars indicate values that were significantly different from that of wild type ($p < 0.01$ for $I_{K}/I_{Na}$ and $p < 0.05$ for $I_{L}/I_{Na}$). Values for wild type and αD602C were taken from Fig. 3 for comparison. Currents were measured in the same manner as for Fig. 3. Data were collected from 5–13 oocytes. Error bars represent S.E.

![Figure 5](image-url)  
**Fig. 5D** (right panel), αD602Kβγ channels displayed inward rectification when bathed in solutions containing Na$^+$ or Li$^+$ as the primary cation, whereas wild type channels did not show obvious rectification (Fig. 5A, right panel). The two mutants (αE595Cβγ and αD602C(βγ) that expressed measurable K$^+$ currents happened to be mutants that expressed very low levels of amiloride-sensitive Na$^+$ current (Fig. 2A). We examined whether the low levels of expressed Na$^+$...
currents compromised the measurements of $I_{K}/I_{Na}$. Injection of a reduced amount of wild type $\alpha\beta\gamma$ mENaC cRNA (0.3 ng/subunit) resulted in expression of amiloride-sensitive inward $Na^{+}$ currents in the range of $0.2-0.6 \mu A$, similar in magnitude to that observed with $\alpha E595C\beta\gamma$ and $\alpha D602C\beta\gamma$. As expected, the $I_{L}/I_{Na}$ and $I_{K}/I_{Na}$ of the wild type mENaC were 1.81 $\pm$ 0.12 ($n = 4$) and $0 \pm 0$ ($n = 4$), respectively, and did not differ from the $I_{L}/I_{Na}$ and $I_{K}/I_{Na}$ we observed for wild type channels from oocytes injected with 2–4 ng of cRNA/subunit. We probed the accessibility of cysteine substitutions at positions $\alpha$Glu$^{595}$, $\alpha$Glu$^{598}$, $\alpha$Asp$^{602}$, or $\alpha$Thr$^{607}$ within mENaC M2 to an externally applied sulfhydryl reagent (MTSET). None of the mutant channels responded to MTSET with a change in amiloride-sensitive whole cell $Na^{+}$ currents that differed significantly from wild type (data not shown). These results suggest that these residues are not accessible to external MTSET, as expected given their location within M2. We cannot exclude the possibility that MTSET reacted with these cysteine residues but resulted in no change in channel activity.

**DISCUSSION**

The last transmembrane domains of most cation-selective ion channels (i.e. S6 or M2) form a component of the channel pore (11–17, 26). Our results suggest that ENaC M2 residues...
contribute to the formation of the pore, based on the findings that selected mutations within M2 alter whole cell currents and cation selectivity.

Negatively charged residues are interspersed in a conserved manner within the M2 domains of ENaC subunits (Fig. 1). Our results indicate that point mutations at any of the three negatively charged residues in the M2 domain of omENaC subunit significantly reduced amiloride-sensitive Na\(^+\) currents, and expression of channels with either double (αβD544C γD562C) or triple (αD602C βD544C γD562C) mutations of the negatively charged residues with ENaC M2 domains failed to produce measurable amiloride-sensitive Na\(^+\) current in agreement with observations of Langlo et al. (9). The reduction in whole cell Na\(^+\) currents observed with point mutations of polar residues within mENaC M2 domains, as well as the reduction in single-channel Li\(^+\) conductance previously reported with αD575Rβγ hENaC (human αAsp\(^{575}\) γAsp\(^{602}\) mouse αAsp\(^{602}\) at equivalent positions within αENaC), are consistent with the notion that these acidic residues may line the conduction pore and their side chains may provide ion-binding sites (9). Alternatively, these charged residues may have a role in maintaining proper channel conformation, and mutations within M2 domains may disrupt or destabilize the conformation of the channel pore, leading to a reduction of ion conduction. Membrane proteins with charged residues within helical transmembrane domains are common and are often neutralized by residues with countercharges within other membrane-spanning domains of the same protein or interacting proteins. For example, positive charges in S4 domains of voltage-gated K\(^+\) channels are thought to be neutralized by negative charges in S2 and S3 domains through ion pairing, providing a mechanism of voltage-dependent channel gating (18, 19). The interdomain electrostatic interactions between transmembrane domains have also been suggested to mediate folding of voltage-gated K\(^+\) channels (20). Mutations that alter the channel conformation may interfere with its assembly or trafficking to the plasma membrane, although Langlo et al. (9) have shown that reversal of the three negatively charged residues within human αENaC does not significantly alter the surface expression of the mutant channels based on confocal images of oocytes expressing mutant αhENaC with green fluorescence protein-tagged β or γ hENaC (9). Reduction of whole cell Na\(^+\) currents observed with mutations within the M2 domain may also reflect changes in channel open probability, although Langlo et al. (9) reported that channel gating is not significantly altered by mutations of negatively charged M2 residues. The M2 domain may have a role in the regulation of channel gating, because ENaC M2 chimeras exhibited alterations in channel open probability (21), and a track within M2 of rat βENaC (Leu\(^{535}\)Glu\(^{540}\)) affected gating properties of αβ γENaC (22). Multiple mechanisms may account for the current reduction we observed with point mutations within mENaC M2 domains. Mutation of a residue within Kir channels (Asp\(^{172}\) of Kir 2.1, Asn\(^{171}\) of Kir 1.1; and Glu\(^{158}\) of Kir 4.1) has been shown to affect ion permeation, selectivity, inward rectification, and sensitivity to channel modulators (23–25).

For many cation-selective ion channels, ion selectivity is largely governed by a selectivity filter that is formed by a conserved sequence of amino acid residues within a pore region (or "P" loop) preceding the M2 or S6 domains (26–28). ENaC has an analogous 3-residue tract (G/S/IXS) preceding M2 within each subunit that has been identified as a key component of the pore region that confers cation selectivity (3–6). Our results suggest that there are other sites beyond the pore region of ENaC where mutations result in measurable amiloride-sensitive K\(^+\) currents. The residues αGlu\(^{595}\) and αAsp\(^{602}\) within M2 may have a role in restricting K\(^+\) permeation through ENaC (Figs. 3–5). Although Langlo et al. (9) did not observe K\(^+\)-permeable channels when arginine was substituted at the corresponding residues within αhENaC, the whole cell Na\(^+\) currents measured in oocytes expressing these mutant hENaCs were very small (46–93 nA at −100 mV) (9), and measurement of even smaller inward K\(^+\) currents would be difficult. Furthermore, our results suggest that multiple residues within M2 of αENaC affect Li\(^+\)/Na\(^+\) selectivity (Figs. 3–5).

How do mutations at αE595 or αD602 within mENaC M2 alter channel selectivity? We previously suggested that the ENaC pore might be arranged in a manner similar to the KcsA K\(^+\) channel pore (6, 7). This would place αGlu\(^{595}\) and αAsp\(^{602}\) in close proximity to the putative selectivity filter formed by (G/S/IXS) track and adjacent residues and allow for side-chain interactions between αGlu\(^{595}\), αAsp\(^{602}\), and selectivity filter residues. These side-chain interactions may have an important role in maintaining the precise structure of the filter. Alternatively, αAsp\(^{602}\) may contribute to a secondary selectivity filter that is located internal to the main selectivity filter. A second "internal" selectivity filter has been proposed for the K\(^+\) channel Kir 2.1, because mutations of Ser\(^{165}\) and Asp\(^{172}\) within the M2 domain of Kir 2.1 alter cation selectivity (15, 24, 29). A point mutation within S6 of the voltage-gated Shaker K\(^+\) channel (A463C) decreased K\(^+\) affinity from the micromolar to millimolar range (30). This residue can be aligned with αAsp\(^{602}\) within the ENaC M2 domain (Fig. 6B). By analogy, mutations of αAsp\(^{602}\) might modify cation ENaC selectivity by altering Na\(^+\) and/or K\(^+\) affinity for a site within the channel pore. Our data cannot distinguish among these possible explanations for K\(^+\) permeation observed with the αGlu\(^{595}\) and αAsp\(^{602}\) mutations. However, homology modeling of αENaC M2 and models of Kir 2.1 M2 (based on the structure of KcsA) suggest that αAsp\(^{602}\) aligns with Ser\(^{165}\) within the M2 of Kir 2.1 (Fig. 6B). We propose that αAsp\(^{602}\) is a pore-lining residue and a ring of four aspartate residues (2 from α, 1 from β, and 1 from γENaC) form an additional cation binding site (or secondary selectivity filter) in the ENaC pore. Although mutation of the corresponding residues within βmENaC (βD544C) or γmENaC (γD562C) did not result in K\(^+\)-permeable channels, γD562C significantly increased the Li\(^+\)/Na\(^+\) current ratio. The absence of K\(^+\) permeation through channels containing βD544C or γD562C, and K\(^+\) permeation through αD602Cβγ, may reflect the channel subunit stoichiometry (i.e. 2α, 1β, 1γ) (31, 32).

In contrast to wild type mENaC, whole cell currents measured in oocytes expressing αD602Kβγ displayed voltage dependence (inward rectification) when oocytes where bathed with either Li\(^+\) or Na\(^+\) (Fig. 5D). Amiloride-inhibitable inward currents were not observed with clamping voltages up to +60 mV. Interestingly, the introduction of a cysteine residue at αAsp\(^{602}\) did not induce a similar voltage-dependence (Fig. 5C). The current rectification observed with αD602Kβγ is reminiscent of the inward rectification observed with Kir 2.1, where Asp\(^{172}\) serves as blocking site for intracellular Mg\(^{2+}\) (33–35). The introduction of positively charged lysine or histidine at Asp\(^{172}\) within Kir 2.1 results in permanent rectification (36, 37). It is possible that the introduction of a positive charge at αAsp\(^{602}\) of mENaC caused inward rectification of Li\(^+\) and Na\(^+\) currents as a result of blocking outward currents due to electrostatic interaction between the charged amino group of αD602K and permeant Li\(^+\) or Na\(^+\). Residue αD602K may reorientate its side chain toward extracellular space during depolarization, and as a result the pore diameter at this site is slightly reduced. Outward ion flow therefore is blocked while inward ion flow occurs at negative membrane potentials. Al-
enlarged opening of the selectivity filter (15). Mutation A563C in Shaker is also significant, and it may have a role in conferring ion selectivity.

All these domains may work together in a concerted manner to achieve the unique cation-selective profile of ENaC.

On the basis of secondary structural predictions and mutagenesis studies, it is likely that ENaC M2 domains are α-helical. Whether the M2 helices are arranged similarly to the resolved M2 structures within the other two transmembrane domain ion channels is unclear. In KcsA K⁺ channel, M2 domains consisting of 27 residues traverse the membrane at an angle of 25° relative to the membrane normal. Their amino termini are packed against pore helices and the selectivity filter, and carboxyl termini form the inner vestibule of the channel pore (26). The S6 helices of voltage-gated K⁺ channels have a conserved Pro-X-Pro motif near the carboxyl terminus that induces a kink in the helix (45). ENaC M2 domains lack proline residues, and helical wheel analyses of ENaC M2 domains indicate that all polar M2 residues align with one face of the helix. We propose that ENaC M2 domains exist as straight helices without a kink. Furthermore, the changes in selectivity observed with mutations at αAsp⁶⁰² suggest that the ENaC M2 residues may be located in close proximity to the pore axis, thus forming a second selectivity or cation-binding site. A structural model for the ENaC M2 is illustrated in Fig. 6A. The two ENaC M2 helices face each other and are tilted with respect to the membrane normal, forming an “inverted teepee” shape, similarly to KcsA. The three charged residues within the ENaC M2 face the pore. In summary, our results suggest that residues within the M2 domain of ENaC contribute to the conduction pore and that, in addition to the selectivity filter preceding M2, selected sites within M2 (αGlu⁵⁹⁶ and αAsp⁶⁰²) may have a role in conferring ion selectivity.

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alternatively, αD602K may enhance the voltage dependence of ENaC open probability. It has been demonstrated that ENaC open probability increases during hyperpolarization (38, 39).

Further studies are required to elucidate the behavior of the current-voltage relationship of the mutant channel.

Although the resolved structure of KcsA K⁺ channel pore revealed an elegant explanation for the mechanism of cation selectivity, the ion selectivity process may not be accomplished exclusively by the selectivity filter. Mutations within K⁺ channel S6 or M2 domains affect ion selectivity, unitary conductance, gating, and inhibition by cytoplasmic blockers. Recent studies have shown that non-hole region domains may also contribute to the ion selectivity process (15, 40–42), suggesting that mechanisms of ion selectivity may be more complex than simple interactions between permeant ions and several key residues forming the selectivity filter. Mutations outside of the pore region affect cation selectivity of ENaC, suggesting ENaC selectivity may involve multiple sites. Two of arginine residues (K504E and K515E) near the carboxyl terminus of the extracellular domain of bovine αENaC alter Na⁺/K⁺ selectivity, amiloride sensitivity, and gating behavior (43). Mutations of arginine residues (αR586E–R587E) near the carboxy-terminal end of human αENaC M2 also resulted in significant changes in K⁺/Na⁺ and Li⁺/Na⁺ selectivity (10). A stretch of residues within the amino terminus (preceding the first transmembrane domain) of an acid-sensing ion channel type 2, a member of the ENaC/degenerin family, was identified as a region that has a role in determining K⁺/Na⁺ selectivity (44). All these domains may work together in a concerted manner to achieve the unique cation-selective profile of ENaC.

Fig. 6. Structural models of the αENaC M2 domains and sequence alignments with M2 domains or S6 domains of other cation channels. A, a structural model was generated by building two α-helices from residues Val⁵⁹⁶–Arg⁶¹⁶ of αENaC M2 using HyperChem 6.03 (Hypercube Inc., Gainesville, FL). The model is presented as a stick model with ribbon rendering of the backbone shown with yellow lines. The displayed and labeled side chains indicate mutations at these sites resulted in change in Li⁺/Na⁺ and/or K⁺/Na⁺ current ratios whereas other residues are not displayed. Residues Glu⁵⁹⁵ and Asp⁶⁰² are indicated in red to show mutations at these locations that made the mutant channel K⁺ permeable. The two helices are tilted at an angle relative to the membrane normal, similar to KcsA. Energy minimization was not performed. Element colors are as follows: cyan for carbon, dark blue for nitrogen, dark yellow for sulfur, and red for oxygen. B, proposed sequence alignments between αENaC M2 domain and the M2 or S6 domains of other cation channels were performed by aligning αAsp⁶⁰² (in red) of αENaC with Ser⁶⁰⁶ (in red) of Kir 2.1 and Ala⁶⁰⁶ (in red) of Shaker B. Substitution of Ser⁶⁰⁶ in Kir 2.1 with a leucine abolished Rb⁻ blockage and converted the channel from highly K⁺-selective against Rb⁻ (Iₚ/Rb⁻ = 0.08) to poorly selective between K⁺ and Rb⁻ (Iₚ/Rb⁻ = 1.23) (15). Mutation A563C in Shaker B decreased internal K⁺ affinity of the channel by ~1000-fold, and large Na⁺ currents were observed in the absence of K⁺. Residues within αENaC M2 where mutations altered Li⁺/Na⁺ current ratios are underlined. αAsp⁶⁰² and αAsp⁶⁰² in αENaC are highlighted to indicate residues where a charge reversal of both side chains increased the K⁺/Na⁺ current ratio (10). Highlighted residues in Kir 2.1 and Shaker B were proposed to expose their side chains to the conducting pore based on the accessibility to the sulfhydryl reagent MTSET (12, 46, 47).

KcsA residues buried behind the selectivity signature sequence TVGYG and the pore helix are boxed. The boldface residues in KcsA extend their side chains to the pore in the resolved structure (28). Sequences in the alignments are, αENaC (GenBank™ accession number AF112185); αENaC (GenBank™ accession number L29007); Kir 2.1 (mouse inward rectifier K⁺ channel type 2; Swiss-Prot accession number P35561); KcsA (Protein Information Resource accession number S60172); and Shaker B (EMBL accession number X06742).
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Shaohu Sheng, Kathleen A. McNulty, Johanna M. Harvey and Thomas R. Kleyman

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