Conserved cysteines in the finger domain of the epithelial Na⁺ channel α and γ subunits are proximal to the dynamic finger–thumb domain interface

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ABSTRACT
The epithelial Na⁺ channel (ENaC) is a member of the ENaC/Degenerin family of ion channels. In the structure of a related family member, the ‘thumb’ domain’s base interacts with the pore, and its tip interacts with the divergent ‘finger’ domain. Between the base and tip, the thumb domain is characterized by a conserved 5-rung disulfide ladder holding together two anti-parallel α helices. The ENaC α and γ subunits’ finger domains harbor autoinhibitory tracts that can be proteolytically liberated to activate the channel, and also host an ENaC-specific pair of cysteines. Using a crosslinking approach, we show that one of the finger domain cysteines in the α subunit (αC263) and both of the finger domain cysteines in the γ subunit (γC213 and γC220) lie near the dynamic finger–thumb domain interface. Our data suggest that the αC256/αC263 pair is not disulfide bonded. In contrast, we found that the γC213/γC220 pair is disulfide bonded. Our data also suggest the γ subunit lacks the terminal rung in the thumb domain disulfide ladder, suggesting asymmetry between the subunits. We also observed functional asymmetry between the α and γ subunit finger–thumb domain interfaces: crosslinks bridging the α subunit finger–thumb interface only inhibited ENaC currents, while crosslinks bridging the γ subunit finger–thumb interface activated or inhibited currents dependent on the length of the crosslinker. Our data suggest that reactive cysteines lie at the dynamic finger–thumb interfaces of the α and γ subunits, and may play a yet undefined role in channel regulation.

INTRODUCTION
The epithelial Na⁺ channel (ENaC) facilitates Na⁺ transport in the aldosterone-sensitive distal nephron, the respiratory tract, and the colon (1–4). ENaC-mediated Na⁺ reabsorption in the distal nephron is critical to K⁺ secretion, and plays a key role in extracellular fluid volume homeostasis and blood pressure maintenance (1). ENaC also plays sensory roles in the taste buds, vascular endothelia and smooth muscle, and the brain (5). At the luminal surface in its varied roles, ENaC is exposed to dynamic extracellular environments and is responsive to stimuli in those environments, including Na⁺, Cl⁻, H⁺, and shear stress. These factors directly regulate the channel by influencing the channel’s open probability (6).

ENaC is a member of the ENaC/Degenerin family of ion channels, which are cation selective channels that gate in response to extracellular cues (6). There are four ENaC subunits, α, β, γ, and δ that assemble to form either αβγ or αβγ heterotrimers. Channel assemblies that include ENaC subunits and acid-sensing ion channel (ASIC) subunits have also been reported (7–9). Sequence comparisons between ENaC subunits and ASIC1, whose structure has been reported, suggest that the core folds of the
proteins are similar (palm and β-ball domains in Fig. 1A), but that the peripheral helical domains (finger, thumb, and knuckle in Fig. 1A) are divergent and may underlie functional differences between the channels (10, 11). The sites of channel activating proteolytic cleavage in the α and γ subunits map to their respective finger domains (6, 12, 13), underlining the notion that the divergent domains are functionally important. We previously reported a model of the α subunit extracellular domains using ASIC1 homology complemented by constraints for the divergent finger domain derived from functional experiments (10).

ENaC/degenerin proteins contain 14 conserved cysteines in the extracellular domains (11, 14). Based on functional experiments, Sheng and colleagues proposed a model for the α subunit where 10 of the cysteines formed a disulfide bond ladder that defined one domain, and the remaining 4 cysteines formed two disulfide bonds elsewhere in the structure (14). The ASIC1 structure accorded with these results, and showed that the disulfide bond ladder held two helices together in an antiparallel fashion and formed the core of the thumb domain (Fig. 1A) (11). Firsov et al. also functionally examined the extracellular domain cysteines, and proposed asymmetry between the subunits regarding the cysteine pair at the top of the thumb domain (15). The thumb domain of each subunit interacts with the finger domain at one end and the extracellular entry to channel pore at the other. Each ENaC subunit finger domain contains two cysteines that are unique to ENaC subunits in the protein family (Figs. 1A, B: Cys-2 and Cys-3), and two additional cysteines that are unique to ENaC β subunits (Fig. 1B). Although we assumed the finger domain cysteines to be disulfide bonded in our α subunit homology model, the disposition of these cysteines had not been experimentally determined.

Double cleavage of the ENaC α and γ subunits liberates autoinhibitory tracts from each subunit (13, 16). Synthetic peptides corresponding to each of the liberated tracts inhibit the channel (17, 18). We have shown that the α subunit-derived inhibitory peptide and the autoinhibitory tract from which it is derived likely bind the α subunit at overlapping sites (10, 19). One key feature of these sites is involvement of the finger–thumb domain interface. We hypothesized that α subunit cleavage activates the channel by releasing an interaction between the finger and thumb domains. We proposed that this conformational change propagates through the thumb domain to the transmembrane helices that form the channel pore (see Fig. 1A). Indeed, directly crosslinking the finger–thumb interface inhibited the channel in a crosslinker-length dependent manner (20).

Here, through crosslinking experiments we explore the finger–thumb domain interfaces of the α and γ subunits. We show that both interfaces are dynamically linked to channel activity. We also demonstrate that the conserved cysteines in the finger domains of the α and γ subunits (Cys-2 and Cys-3) lie close to the respective finger–thumb domain interfaces. However, our data suggest that while the γ subunit finger domain cysteines are likely disulfide bonded, the α subunit equivalents are not. Additionally, our data suggest that the γ subunit harbors a pair of thumb domain cysteines at the finger–thumb domain interface that are not likely disulfide bonded. These data suggest that reactive cysteines lie at the dynamic finger–thumb interfaces of the α and γ subunits.

RESULTS

Finger domain cysteines in the α subunit are near the finger–thumb domain interface

A peptide derived from the furin-released autoinhibitory fragment of the α subunit decreased channel currents by binding at the finger–thumb domain interface of the α subunit (19). Previously, we recapitulated this effect in the absence of the inhibitory peptide by constraining the distance between the finger domain (using residue 171 in helix 1, red circle in Fig. 1A) and the thumb domain (blue circle in Fig. 1A) of the α subunit using short MTS-based crosslinkers (20). Inhibition by MTS crosslinkers was length-dependent.
To further examine the dynamics at the finger–thumb domain interface, we aimed to induce a direct disulfide bridge between cysteines introduced at positions 171 and 474 in the α subunit using the oxidizing agent Cu-Phe (21). However, when we applied 4 μM Cu-Phe for 15 s to the αY474C single mutant co-expressed with wild type β and γ subunits in Xenopus oocytes, we observed a modest inhibition of channel currents (Fig. 2). Cu²⁺ alone had no effect on αY474C currents (Fig. 2A, cyan traces), reducing currents by 5±2% (n=7, p=NS by paired t-test). Inhibition by Cu-Phe reversed only upon addition of the reducing agent DTT (Fig. 2A), suggesting a covalent modification of the channel. This contrasted with effects on wild type currents, which were unaffected by 4 μM Cu-Phe or 4 μM Cu²⁺ addition. The absence of an effect with Cu²⁺ alone is consistent with previous work (22). Notably, extracellular DTT treatment did not affect wild type currents, consistent with previous studies (20, 23, 24). Wild type channel insensitivity to DTT suggests either that native extracellular disulfide bonds are not susceptible to reduction by DTT, or that reduction of those bonds does not affect channel function. Although Cu-Phe-induced disulfide bonds may also be DTT-insensitive, DTT-reversal after oxidation in the case of αY474C suggests reduction of an induced disulfide bond. We also found that Cu-Phe had no effect on neighboring αK476C mutant channels, showing that the Cu-Phe effect is specific to αY474C (Figs. 2A, C).

We hypothesized that Y474C formed a disulfide bond with an endogenous cysteine in the presence of Cu-Phe. ENaC subunits have cysteine pairs that correspond to all 7 disulfide-bridged pairs observed in the extracellular domains of the ASIC1 structure (Fig. 1). Functional experiments suggest that these cysteines may be similarly disulfide bonded in ENaC subunits (14, 15). ENaC subunits have an additional pair of conserved cysteines in their finger domains (Figs. 1A, B: Cys-2 and Cys-3). The disulfide bonding status and position of the finger domain cysteine pair is unknown, although our α subunit homology model posited C256 (αCys-2) and C263 (αCys-3) in a disulfide bond and placed them more than 15 Å from Y474 (10). To test whether Cu-Phe induced a bond between αY474C and either of the α subunit finger domain cysteines, we mutated each to alanine in the background of αY474C, and treated oocytes expressing the double-mutants with 4 μM Cu-Phe. We found that Cu-Phe inhibited αC256A,Y474C currents in a DTT reversible manner, similar to what we observed with the αY474C single mutant, and different from wild type. In contrast, any Cu-Phe effect on αC263A,Y474C currents reversed upon washout of the oxidant, and DTT had no effect on measured currents. We note here that we frequently observed Cu-Phe inhibition that reversed upon washout prior to DTT addition in each of the groups tested. This suggests an unstable conformational change may be induced by Cu-Phe, but this effect is difficult to interpret. These results show that DTT-reversible Cu-Phe inhibition of αY474C requires endogenous αC263. These results suggest that αC263 is in close proximity to the finger-thumb domain interface, and is free to form a disulfide bond in the presence of αY474C.

Crosslinking across the γ subunit finger-thumb domain interface potentiates ENaC currents

Sequence homology suggests significant structural and functional symmetry between ENaC subunits (25). Yet functional differences are clearly evident. For example, the α and γ subunits are subject to activating proteolysis, while the β and δ subunits are not (12, 26, 27). We hypothesized that the conformations of the channel pore and the γ subunit’s finger-thumb domain interface were coupled, analogous to the α subunit (20). We introduced a cysteine at position 115 in the γ subunit (γD115C), which is equivalent to site 171 in helix 1 of the α subunit at the finger-thumb domain interface (see Fig. 1A). We previously reported evidence of crosslinking between this site and Y474C at the top of the thumb domain in the α subunit (20). When we perfused bifunctional MTS-2-MTS for 1 min,
we observed an increase in channel currents that remained elevated after washout of the crosslinker (Fig. 3A). DTT appeared to slowly reverse the activation, although its slow kinetics made it difficult to distinguish it from channel run-down. Furthermore, the effect was dependent on the length of the crosslinker. Neither MTS-1-MTS nor MTS-3-MTS had an effect on γD115C currents (Fig. 3B). In contrast, 1 min treatment of each of these MTS crosslinkers irreversibly inhibited wild type ENaC currents by an average of 24%, and in each case was different from the mutant tested with the same crosslinker. We and others have previously reported this effect on wild type ENaC, and that it is attenuated by amiloride (20, 23, 28). Our data showing MTS-2-MTS activation of γD115C in contrast to inhibition of wild type channels suggest covalent modification of γD115C. We hypothesized that the length dependent activation and DTT reversibility observed with γD115C resulted from crosslinking between γD115C on helix 1 in the finger domain and an endogenous cysteine.

To test our hypothesis, we examined endogenous cysteines that may be nearby. The γ subunit finger domain has 2 cysteines, γC213 (γCys-2) and γC220 (γCys-3), that are conserved among ENaC subunits (Figs. 1A, B). To identify the putative endogenous cysteine required for γD115C activation by MTS-2-MTS, we tested γD115C channels where candidate cysteines were mutated to alanine (Fig. 4A). When we tested both γD115C,C213A and γD115C,C220A, we found that MTS-2-MTS activated both channels similarly to MTS-2-MTS activation of γD115C (Figs. 4B, C). γD115C lies on helix 1 in the finger domain at the finger–thumb domain interface (see Figs. 1A, 4A). The thumb domain has 4 cysteines that may form 2 disulfide pairs near the finger–thumb domain interface (Figs. 1A, C) (11, 14, 15). In the mouse ENaC γ subunit, the two thumb domain pairs are γC413/γC440 (γCys-10/Cys-13) and γC415/γC429 (γCys-11/Cys-12). Firsov et al. suggested that γC415 and γC429 may not be disulfide bonded (15). We tested γD115C channels bearing alanine mutations at each of these sites with MTS-2-MTS. We found that γD115C,C415A channels were not activated by MTS-2-MTS, and that the MTS-2-MTS effect was different than γD115C (Fig. 4C). In contrast, γD115C,C429A and γD115C,C440A channels were activated by MTS-2-MTS, but that activation was attenuated compared to γD115C channels. We could not test γD115C,C413A due to poor functional expression. Our results show that MTS-2-MTS activation of γD115C requires endogenous γC415. The results of these experiments suggest that in the presence of γD115C, γC415 is free and in close proximity to γD115C.

Endogenous γ subunit finger domain cysteines are near the finger–thumb domain interface

In the course of performing the experiments shown in Fig. 4, we observed that both the γC213A (γCys-2) and γC220A (γCys-3) single mutants responded to bifunctional MTS crosslinkers differently than wild type (Fig. 5). Whereas wild type currents were consistently inhibited by each of the MTS crosslinkers tested and were not DTT sensitive, currents from γC213A and γC220A channels both responded in a crosslinker-length dependent manner (Fig. 5, Table 1). MTS-1-MTS had little effect on γC213A and γC220A channels. MTS-2-MTS stimulated currents from both mutant channels, which reversed upon DTT addition. This potentiation was in stark contrast to MTS-2-MTS inhibition of wild type channels. MTS-4-MTS had no effect on either mutant channel; this non-effect was also different from MTS-4-MTS inhibition of wild type channels. MTS-8-MTS inhibited both γC213A and γC220A in a DTT insensitive manner, reminiscent of the effect of MTS-8-MTS on wild type channels. Clearly, the effects of the shorter MTS crosslinkers were length-dependent for γC213A and γC220A (see Table 1) and slowly reversed with DTT. These data suggest that the shorter MTS compounds bridged the remaining γ subunit finger domain cysteine and a nearby endogenous cysteine, while MTS-8-MTS may have inhibited mutant channel currents analogously to wild type.
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channels. Our data suggests that γC213 and γC220 are ordinarily disulfide bonded, but that when either is mutated to alanine, the other becomes capable of crosslinking to an endogenous cysteine. In support of this notion, when we mutated both γC213 and γC220 to alanine, the current response to both MTS-2-MTS and MTS-8-MTS reverted to wild type (Fig. 5).

We hypothesized that like the α subunit finger domain cysteines, the γ subunit finger domain cysteines lie near the finger–thumb domain interface, and that MTS-2-MTS activation of both γC213A and γC220A mutant channels may require residue γC415, or possibly γC429. In the background of each single mutant, we mutated either γC415 or γC429 to alanine. We then tested the four double mutants with each of the bifunctional MTS crosslinkers (Fig. 5). MTS-2-MTS did not activate currents from γC213A,C415A, in clear contrast to MTS-2-MTS activation of the corresponding single mutant. None of the four double mutants exhibited differences between the effects of MTS-1-MTS, MTS-2-MTS, and MTS-4-MTS, in contrast to the γC213A and γC220A single mutants (Fig. 5, Table 1). Further, γC213A,C415A and γC220A,C415A double mutants had attenuated responses to MTS-8-MTS compared to the corresponding single mutant. These data suggest that although MTS-8-MTS inhibition of γC213A and γC220A was not DTT reversible, the effect requires endogenous γC415. On its own, mutation of γC415 to alanine did not change the effects of MTS-2-MTS or MTS-8-MTS on the channel (data not shown). Altogether, our results show that the crosslinker effects on the endogenous γ subunit finger domain cysteine mutants (γCys-2/Cys-3) depend on the presence of endogenous γC415 (γCys-11) at the top of the thumb domain.

**MTS compound reduction of wild type ENaC currents and effects on finger-thumb interface mutants are separate phenomena**

If inhibition of wild type ENaC by MTS crosslinkers was due to modification of a pore cysteine that blocks the pore, as we and others have proposed (20, 23, 28), we would not expect that mutants far from the pore could negate that inhibition. Introduction of a free cysteine at the γ subunit finger-thumb domain interface does exactly that, either through introduction of a novel cysteine (γD115C) or through mutation of one-half of a putative disulfide pair (γC213A or γC220A) (Figs. 3 and 5). This suggests an alternate mechanism underlies bifunctional MTS compound inhibition of wild type ENaC. To test whether residues that are involved in MTS compound-mediated inhibition of wild type ENaC overlap with residues involved in activation in the cases of γC213A and γC220A, we performed a protection experiment (Fig. 6). Prior to 1 min treatment with MTS-2-MTS, we treated wild type and mutant channels with monofunctional MTS-2 for 1 min, or alternatively with vehicle. While mock pre-treated mutants were activated by MTS-2-MTS as seen before in Fig. 5, MTS-2 pre-treatment attenuated activation by MTS-2-MTS for both γC213A and γC220A by 61% and 81%, respectively. In contrast, MTS-2 pretreatment of wild type channels had no effect on subsequent inhibition by MTS-2-MTS (Figure 6). These data show that MTS-2 protects the γC213A and γC220A mutants from reaction with MTS-2-MTS, but that MTS-2 cannot protect wild type ENaC from irreversible inhibition by MTS-2-MTS. These data suggest that the mechanisms underlying wild type channel inhibition and γC213A or γC220A activation by MTS compounds are distinct.

**Mutation of finger–thumb domain interface cysteines reduces channel expression at the cell surface**

We observed reduced currents for channels with mutated finger–thumb domain interface cysteines. Changes in whole cell currents may reflect changes in the number of channels at the cell surface and/or the biophysical characteristics of those channels. To determine whether these mutations affected the number of channels at the cell surface, we incorporated a β-subunit with an extracellular FLAG tag to perform a chemiluminescence-
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based assay. One day after cRNA injection of oocytes, we measured amiloride-sensitive currents by two-electrode voltage clamp at -100 mV (Fig. 7A). Currents were greatly reduced for \( \alpha C256A, Y474C \) and \( \alpha C263, Y474C \), moderately reduced for \( \gamma C415A \) and \( \gamma C429A \), and modestly reduced for \( \gamma C220A \). Two days after cRNA injection, we used an anti-FLAG antibody to quantify channels on the cell surface (Fig. 7B). Surface expression measurements largely paralleled current measurements. Similar to the effect on currents, \( \alpha C256A, Y474C \) and \( \alpha C263, Y474C \) exhibited the greatest reductions in surface expression. Surface expression of \( \gamma C213A \) was modestly reduced where currents were unchanged, and surface expression of \( \gamma C429A \) was unchanged where currents were moderately reduced. These data suggest that changes in cell surface expression largely accounts for the differences in currents we observed, but that there are likely differences at the single channel level as well.

**Finger–thumb domain interface cysteines do not mediate inhibition by SIN-1, a peroxynitrite generator**

Our data suggests that neither \( \alpha C256/\alpha C263 (\alpha Cys-2/Cys-3) \) in the finger domain, nor \( \gamma C415/\gamma C429 (\gamma Cys-11/Cys-12) \) in the thumb domain are disulfide bonded. That their modification can modulate channel activity raises the possibility that oxidants or reactive oxygen species can directly regulate ENaC through these sites. Reactive oxygen species have been shown to regulate ENaC activity (29–31). DuVall et al. reported that peroxynitrite produced from SIN-1 inhibited currents from ENaC expressed in oocytes (32). SIN-1 reacts at physiological pH to produce nitric oxide and superoxide, which subsequently react to form peroxynitrite. SIN-1 treatment of oocytes expressing ENaC rapidly inhibited amiloride sensitive currents (76 ± 16 %) (Figs. 8A, B). Subsequent removal of SIN-1 from the perfusate rapidly restored currents to 77 ± 12 % of their starting values (Figs. 8A, C). We then tested alanine mutants of \( \alpha C256, \alpha C263, \gamma C415, \) and \( \gamma C429 \) to determine whether SIN-1 dependent inhibition required cysteine residues at any of these sites. For each of the mutants tested, the reduction of current in the presence of SIN-1 was similar to wild type. Neither did any of the mutants tested reduce the sustained inhibition observed after SIN-1 removal. On the contrary, \( \alpha C256A \) increased inhibition observed after SIN-1 removal, compared to wild type. Our data suggest that SIN-1 dependent ENaC inhibition does not require any of the cysteines tested here.

**DISCUSSION**

Two groups previously identified several disulfide pairs in the extracellular domains of the \( \alpha, \beta, \) and \( \gamma \) subunits using a double mutant-cycle approach (14, 15). According with these results, the structure of homologous ASIC1 showed that the 14 extracellular cysteines that are conserved between ENaC and ASIC subunits formed 7 disulfide bonds (11). ENaC subunits have a unique pair of cysteines in the divergent finger domain. Experiments by Sheng et al. to determine whether the conserved finger domain cysteines are disulfide bonded to each other were either inconclusive or unsupportive of the presence of such a bond (14). Regarding the \( \gamma C415/\gamma C429 (\gamma Cys-11/Cys-12) \) pair, previous experiments suggested a disulfide bond for the equivalent pairs in the \( \alpha \) (14, 15) and \( \beta \) subunits (15). However, neither study concluded that \( \gamma C415 \) and \( \gamma C429 \) are disulfide bonded. Indeed, Firsov et al. noted functional asymmetry regarding this cysteine pair in the \( \gamma \) subunit (15).

For the \( \alpha \) subunit, our results suggest that \( \alpha C263 (\alpha Cys-3) \) is near the finger–thumb domain interface, and is free to form a disulfide bond with an introduced cysteine. We cannot rule out the possibility that \( \alpha C256 (\alpha Cys-2) \) and \( \alpha C263 (\alpha Cys-3) \) are a disulfide pair in the absence of an introduced cysteine in the \( \alpha \) subunit finger domain, our results suggest that \( \gamma C213 (\gamma Cys-2) \) and \( \gamma C220 (\gamma Cys-3) \) are disulfide bonded. The removal of one by mutagenesis renders channel currents susceptible to cysteine reactive MTS reagents.
Furthermore, our data suggest that γC415 (γCys-11) and γC429 (γCys-12) are not disulfide bonded, at least in the context of the γD115C, γC213A, or γC220A mutants, and in accord with earlier studies (14, 15). This was somewhat surprising because earlier studies were ambiguous on the status of the γC415/γC429 pair, these residues are conserved across the protein family, and the equivalent sites in ASIC1 are disulfide bonded (11). This finding suggests that thiol reactive molecules could directly regulate the channel via an available cysteine near the finger–thumb interface of either the α or γ subunits. We tested SIN-1, a nitric oxide and peroxynitrite generator that has been reported to inhibit ENaC currents in oocytes (32, 33). We found that inhibition rapidly reversed when we removed SIN-1, suggesting either a non-covalent interaction or an unstable modification of the channel by SIN-1 or its decomposition products. We also found that none of the cysteines at the finger–thumb domain interface that our data suggest are free were required for SIN-1 dependent inhibition. We also observed functional asymmetry between the α and γ subunits. We previously reported that constraining the finger–thumb interface of the α subunit using short bifunctional MTS crosslinkers inhibited ENaC (20). In contrast, here we show that constraining the finger–thumb interface through a number of different sites on the γ subunit finger domain using a short bifunctional MTS crosslinker activated ENaC. The disparity may reflect important differences in the experiment, e.g., involvement of endogenous cysteines in the γ thumb domain that are close but do not align with the thumb domain site we used in the α subunit. Alternatively, equivalent movements at the finger–thumb interface in the α and γ subunits may not have equivalent effects on the conformation of the channel pore. For example, the γ subunit’s TM1–β1 linker, which may transduce conformational changes from the thumb domain to the pore (see Fig. 1A), is two residues shorter than those of either the α or β subunits. We previously used normal mode analysis to examine motions in the ectodomains that resonated with gating-like motions in the pore (10). Despite using a symmetric α3 ENaC homology model with or without the autoinhibitory tract in all subunits, we observed asymmetry in the finger and thumb domains. Plainly, a channel comprised of distinct but related subunits could give rise to functional asymmetry. Other ENaC functional properties demonstrate asymmetry as well, including proteolytic activation (27), Cl− inhibition (34), Na+ self-inhibition (35, 36) and those relating to the pore (37, 38).

We have revised our model of the ENaC α subunit extracellular domains to account for our data. Our results suggest that αC263 and αC256 are not disulfide bonded. Our data also suggest that αC263 (αCys-2) lies close to αY474 in the finger–thumb domain interface, in contrast to our previous model. Our earlier model was based on homology to ASIC1 with the exception of the finger domain (10). We constrained the finger domain using predicted secondary structure and constraints derived from functional experiments examining inhibition by an α subunit-derived inhibitory peptide. These included scanning mutagenesis and double-mutant cycle experiments. We have subsequently described crosslinking experiments here (Fig. 2) and in previous reports (10, 20, 24). These allow for a straightforward derivation of new structural constraints. We have included these (Table 2) and generated a new model of the ENaC α subunit extracellular domains using Modeller (39). Several of the new constraints provide more specificity than those based on scanning mutagenesis. As a result, we removed previously derived constraints that included residues E174, S269, and S270, leaving a total of 49 experimentally derived constraints. We generated 20 models (Fig. 9), and scored each by summing the violation distances of experimentally derived constraints (Fig. 9B). The best scoring models satisfy nearly all the constraints we imposed, with 9 models exhibiting less than 1 Å of cumulative violations. Superimposing the models using backbone Ca atoms outside of the finger domain illustrates that the structure of the
finger domain is better constrained near the finger–thumb interface that defines the N-terminal end of the inhibitory peptide binding pocket as compared to the C-terminal end of the peptide binding pocket (Fig. 9A), or residues near either of the furin cleavage sites. Our best scoring model places αC263 close to αY474 (Figs. 9C, D), consistent with our data (see Fig. 2).

In summary, our data suggest that the finger domain cysteines in both the α and γ subunits lie close to the finger–thumb domain interface. In the α subunit, they are likely free. In the γ subunit, the terminal pair in the thumb domain cysteine ladder is likely not disulfide bonded. These free cysteines lie at dynamic domain interfaces and may play a yet undefined role in channel regulation. In contrast to constraining the α subunit finger–thumb domain interface, constraining the γ subunit finger–thumb interface activated the channel, suggesting important dynamic differences between the subunits.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Site-Directed Mutagenesis—**pBluescriptSK (-) (Stratagene California, San Diego, CA) vectors containing mouse α, β, or γ mouse ENaC subunits were previously described (40). Site-directed mutagenesis was performed with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Primers were designed using QuikChange Primer Design (Agilent, Santa Clara, CA) and were obtained from Integrated DNA Technologies (Coralville, IA) to modify mouse ENaC subunits. Direct sequencing was used to confirm constructs.

**ENaC functional expression in Xenopus oocytes—**Plasmids were linearized with restriction endonucleases (New England Biolabs, Ipswich, MA) at 37°C overnight and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Linearized plasmids were transcribed into RNA using mMessage mMachne T3 Transcription Kit (Invitrogen, Carlsbad, CA) and purified using the RNeasy MiniElute Cleanup Kit (Qiagen). Oocytes from *Xenopus laevis* were harvested and defolliculated using type II collagenase (Sigma-Aldrich, St. Louis, MO). 1-4 ng of RNA per ENaC subunit was injected into stage V or VI *Xenopus* oocytes using a Nanoject II (Drummond, Broomall, PA). *Xenopus* oocytes were stored in modified Barth’s saline at 18°C. The protocol for harvesting oocytes from *Xenopus laevis* was approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee.

**Measurement of ENaC Currents—**ENaC current was measured 20-30 hours post-injection using the two-electrode voltage clamp technique using a GeneClamp 500B voltage clamp amplifier (Molecular Devices, Sunnyvale, CA) and pClamp 10.2 software (Molecular Devices). A 20-µl recording chamber (AutoMate Scientific, Berkeley, CA) was used with perfusion (3-5 ml/min) controlled by an eight-channel pinch valve system (AutoMate Scientific). Measurement of ENaC currents were made in Na-110 buffer (110mM NaCl, 2mM KCl, 2mM CaCl₂, and 10mM HEPES (pH 7.4)). At the end of each experiment, currents were measured in 10µM amiloride (stock solution of 100mM amiloride in dimethyl sulfoxide (DMSO) stored at room temperature diluted into Na-110 buffer) to determine the amiloride sensitive component of the current. This was defined as the ENaC-mediated current.

**Crosslinking during ENaC Current measurements—**Stock solutions of methanethiosulphonate (MTS) reagents (Toronto Research Chemicals, North York, ON) were prepared in DMSO at 10 mM and stored at -20°C under desiccant for up to six months. MTS stock solutions were diluted into Na-110 buffer for a final concentration of 4 µM CuSO₄ and 16 µM 1,10-Phenanthroline (Sigma-Aldrich) in ethanol were stored at -20°C. Copper phenanthroline (Cu-Phe) solution was prepared on the day of the experiment by diluting CuSO₄ and 1,10-Phenanthroline stocks into Na-110 buffer for a final concentration of 4 µM CuSO₄ and 16 µM 1,10-Phenanthroline.
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1,10-phenanthroline. CuSO₄ solution was prepared by diluting CuSO₄ stock into Na-110 buffer for a final concentration of 4 µM CuSO₄. Stock solutions of 100 mM dithiothreitol (DTT) (Sigma-Aldrich) in Na-110 buffer (stored at -20°C) were diluted to 10 mM DTT in Na-110 buffer on the day experiments were performed.

ENaC cell surface expression—ENaC cell surface expression in oocytes was measured as previously described (41–43). Mutant α or γ subunits were co-expressed with a β subunit with an extracellular FLAG tag. One day following cRNA injection of oocytes, amiloride-sensitive currents were measured. Two days following cRNA injection of oocytes, oocytes were placed on ice and incubated for 30 min in antibiotic-free MBS and 1% bovine serum albumin (MBS/BSA), and then for 1 h with MBS/BSA with 1 µg/ml mouse anti-FLAG antibody (M2; Sigma). After wash with MBS/BSA, cells were incubated for 1h with 1 µg/ml horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, WestGrove, PA). After wash, oocytes were transferred to a 96-well plate. SuperSignal ELISA Femto Maximum Sensitivity Substrate (100 µL; Thermo Scientific, Rockford, IL) was added to each well. Chemiluminescence was quantified using a GloMax-Multi+ Detection System (Promega, Madison, WI). The mean value measured from 4 wells with no cells was subtracted from the measurements of cells with ENaC-injected oocytes.

SIN-1 generated peroxynitrite treatment—We prepared 100 mM 3-morpholino-sydnonimine (SIN-1) (Millipore, Darmstadt, Germany) stock in 10 mM phosphate buffer. We diluted SIN-1 into Na-110 buffer 1 h before recording currents.

Statistical Analyses—Comparisons between groups were performed by ANOVA followed by a Tukey multiple comparison’s test, by Student’s t-test, or by Kruskal-Wallis rank sum test followed by Dunn test for multiple comparisons with p-values adjusted with the Benjamini-Hochberg method, as indicated. We used R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria) or Prism 7 (GraphPad, Inc., La Jolla, CA) to perform statistical calculations. Adjusted values of p<0.05 were considered significant.

Homology Modelling—Modelling was performed using MODELLER version 9.13 (39). We used the ENaC α subunit – cASIC1 alignment previously described (10), and the cASIC1 structural model (pdb code 4NYK). In addition to the constraints we previously used, we added new constraints derived from crosslinking experiments reported here and in previous work (see Table 2) (10, 20, 24). We constrained distances of Cα–Cα pairs using an upper bound constraint dependent on the crosslinking agent used: Cu-Phe, 7.1 Å; MTS-1-MTS, 11.01 Å; MTS-2-MTS, 12.46 Å; MTS-3-MTS, 13.79 Å; MTS-4-MTS, 15.05 Å; MTS-6-MTS, 17.46 Å; MTS-8-MTS, 19.61 Å, calculated using the Molecule Calculator (44). Using our previous model (10) as an initial input, we generated 20 models, which were scored by summing deviations greater than the set distance of the upper bound constraint. We visually inspected the highest scoring models for structural violations (e.g. knots), and selected a high scoring model with minimal violations. Superpositioning models and rmsd calculations were performed using Swiss pdb viewer v.4.1.0 OSX (45). Molecular figures were generated using PyMOL (Schrodinger, LLC, New York, NY).

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CONFLICT OF INTEREST
The authors declare no competing conflicts of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
AUTHOR CONTRIBUTIONS
BMB and OBK conceptualized the study, interpreted results, prepared figures, and drafted and revised the manuscript. BMB performed homology modeling. BMB, XPW and OBK performed experiments, analyzed data, and approved the final version of the manuscript.

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**FOOTNOTES**

Abbreviations used: ENaC, Epithelial Na+ Channel; ASIC, Acid-sensing Ion Channel; Cu-Phe, copper phenanthroline; MTS, methanethiosulfonate; SIN-1, 3-morpholinosydnonimine

**FIGURE LEGENDS**

**Figure 1. ENaC subunit cysteines.** A, Schematic of an ENaC subunit, adapted from (6). Cysteine residues are represented by yellow circles, and disulfide bonds observed the ASIC1 structure are represented by black bars. Conserved aspartic acids εD171 and γD115 are represented by a red circle and αY474 is represented by a blue circle. B, Sequence alignment of finger domain segment hosting conserved cysteines in ENaC subunits. C, Sequence alignment of the thumb domain residues proximal to the finger domain in the ASIC1 structure. Identities are shaded gray. Cysteines are shaded black.
**Figure 2. Conserved αC263 in the finger domain is proximal to the finger–thumb domain interface.** Mutant or wild type α subunits with wild type β and γ subunits were expressed in *Xenopus* oocytes, and currents were measured by two-electrode voltage clamp at -100 mV. Oocytes were treated with 0.5 μM Cu-Phe for 15 s, followed by 110-Na buffer for 60 s and 10 mM DTT for 60-90 s. A, Representative recordings are shown. B, Inhibition by Cu-Phe was determined by comparing the amiloride-sensitive current at the end of Cu-Phe washout to the amiloride-sensitive current prior to Cu-Phe addition. Bars representing the mean of each group are shown. * p<0.05, ** p<0.01 vs. wild type by Kruskal-Wallis rank sum test (p<0.0001) followed by Dunn test for multiple comparisons with p-values adjusted with the Benjamini-Hochberg method.

**Figure 3. Crosslinkers activate γD115C in a length dependent manner.** Wild type or D115C γ subunits were co-expressed with wild type α and β ENaC subunits in *Xenopus* oocytes, and currents were measured by two-electrode voltage clamp at -100 mV. Oocytes were treated with 0.5 μM MTS-n-MTS for 60 s, followed by 110-Na buffer for 60 s, and 10 mM DTT for 90 s. A, Representative recordings are shown. MTS-2-MTS inhibited currents of wild type (p<0.05 by paired Student's t-test), and subsequent DTT addition had no effect. In contrast, MTS-2-MTS addition increased currents of γD115C (p<0.0001 by paired Student's t-test), and subsequent DTT addition reversed the activation (p<0.001 by paired Student's t-test). B, The change in current due to MTS-n-MTS was determined by comparing the amiloride-sensitive current at the end of MTS-n-MTS washout to the amiloride-sensitive current prior to MTS-n-MTS treatment. Bars representing the mean of each group are shown. ** p<0.01, **** p<0.0001 by one-way ANOVA followed by Tukey post hoc analysis.

**Figure 4. Endogenous γC415A is required for crosslinker activation of γD115C.** A, Schematic of γ subunit finger–thumb interface depicting locations of D115C on helix 1 (rectangle), and endogenous cysteines that may be nearby. Dashed lines indicate possible disulfide bonds. B, Smaller schematics indicate which endogenous cysteine was mutated to alanine (open circle). Mutant γ subunits were co-expressed with wild type α and β subunits in *Xenopus* oocytes, and currents were measured by two-electrode voltage clamp at -100 mV. Oocytes were treated with 0.5 μM MTS-2-MTS for 60 s, followed by 110-Na buffer for 60 s, and then 10 mM DTT for 90 s. Representative recordings are shown. C, Inhibition by MTS-2-MTS was quantified by comparing the amiloride-sensitive current at the end of MTS-2-MTS washout to the amiloride-sensitive current prior to MTS-2-MTS treatment. Bars representing the mean of each group are shown. Dashed lines indicating the mean values for the effect of MTS-2-MTS on wild type and γD115C channels from Fig. 3 are shown. * p<0.05, *** p<0.001, **** p<0.0001 vs γD115C by one-way ANOVA followed by Tukey post hoc analysis.

**Figure 5. Endogenous γ subunit finger domain cysteines are near the finger–thumb domain interface.** Mutant γ subunits were co-expressed with wild type α and β subunits in oocytes, and currents were measured by two-electrode voltage clamp at -100 mV. ENaC expressing oocytes were treated with 0.5 μM MTS-n-MTS as indicated for 60 s, followed 110-Na buffer for 60 s, and then 10 mM DTT for 90 s. A, Representative recordings are shown for wild type and each mutant with an accompanying schematic illustrating the endogenous cysteine(s) removed (open circles). B, The effect of MTS-n-MTS was determined by comparing the amiloride-sensitive current at the end of MTS-n-MTS washout to the amiloride-sensitive current prior to MTS-n-MTS treatment. Bars indicating the mean of each group are shown. Data was analyzed by two-way ANOVA, which indicated differences between channels and between crosslinkers (p<0.0001). We compared individual pairs using Tukey’s multiple comparison test. Indicated in (B) are comparisons between γC213A or γC220A and wild type channels with a given MTS compound.
ENaC finger–thumb domain interface cysteines

(in left column), and between double mutants bearing γC415A or γC429A and the corresponding single mutants, γC213A or γC220A, with a given MTS compound (across the top or center rows, respectively). * p<0.05, **** p<0.0001. Comparisons of the effects of different crosslinkers on a given channel are shown in Table 1.

Figure 6. Monofunctional MTS-2 protects mutant channels but not wild type channels from bifunctional MTS-2-MTS. Mutant or wild type γ subunits were co-expressed with wild type α and β ENaC subunits in *Xenopus* oocytes. ENaC currents were measured using two-electrode voltage clamp at -100 mV. Oocytes were mock treated or treated with 10 µM MTS-2 60 s followed by washout with 110-Na buffer for 60 s. Oocytes were then treated with 10 µM MTS-2-MTS for 60 s followed by washout with 110-Na buffer for 60 s. The effect of MTS-2-MTS treatment after mock or MTS-2 treatment was determined by comparing current after washout of MTS-2-MTS to current just prior to MTS-2-MTS addition. Bars indicating the mean are shown. ** p<0.01, **** p<0.0001, by Student’s t-test.

Figure 7. Mutation of finger–thumb domain interface cysteines reduces cell surface expression. Extracellular FLAG-epitope tagged β subunits (βF) were co-expressed with wild type and mutant ENaC subunits, as indicated. A, One day after cRNA injection of oocytes, amiloride-sensitive (10 µM) currents were measured using two-electrode voltage clamp at -100 mV. B, Two days after injection, the remaining oocytes were placed on ice, and anti-FLAG antibodies were used to detect channels at the cell surface by chemiluminescence. Bars indicating the mean for each group are shown. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 vs βFγ by one-way ANOVA followed by Tukey post hoc analysis.

Figure 8. SIN-1 mediated inhibition of ENaC does not require free cysteines at finger–thumb interface of the α or γ subunits. Mutant ENaC subunits were co-expressed with wild type cognate subunits in *Xenopus* oocytes, and currents were measured by two-electrode voltage clamp at -100 mV. Solutions of 1 mM SIN-1 in 110-Na buffer were perfused for 10 min, preceded and followed by brief pulses with 10 µM amiloride. A, Representative recordings are shown. B, Rapid inhibition by SIN-1 was calculated by comparing the steady state current in the presence of SIN-1 to the amiloride sensitive current before adding SIN-1. C, Sustained inhibition was calculated by comparing the steady state current after washing out SIN-1 to the amiloride sensitive current before adding SIN-1. * p<0.05 by one-way ANOVA followed by Dunnett’s post hoc test.

Figure 9. Model of the α subunit extracellular domains. Modeller was used to create a homology model of the ENaC α subunit extracellular domains, using cASIC1 (pdb code 4NYK) as a template. Distance constraints derived from functional data and predicted secondary structure were used to constrain the finger domain (green), which shares 8% sequence identity with cASIC1. A, Overlay of 9 models with less than 1 Å of cumulative distance violations of constraints derived from functional data, shown as cartoon. Models were superposed using non-finger domain residues: palm (gray), β-ball (pink), thumb (yellow), and knuckle (tan). The overlay shows that the highest scoring models were better constrained at the finger–thumb domain interface near the N-terminus of the bound inhibitory peptide (fuchsia, see center panel), and less well constrained near the C-terminus of the bound inhibitory peptide, and near the furin cleavage sites. B, Plot of models sorted by cumulative violation of distance constraints derived from functional data. 9 of 20 models constructed showed less than 1 Å cumulative violations. C, Best scoring model is shown as cartoon, bound peptide is shown as sticks, and disulfide bonds are shown as orange sticks. D, Close up of best scoring model finger–thumb interface shown as cartoon with positions of key residues highlighted and shown as spheres.
Table 1. Crosslinker length–dependent (MTS-n-MTS) effects on γ subunit cysteine mutants near the finger–thumb domain. For each channel in Fig. 5, we compared the effect of using a crosslinker of a given length with all other crosslinkers tested. The number of carbon atoms between MTS functional groups for the tested pair are indicated. Pairwise comparisons were performed by two-way ANOVA followed by Tukey’s post hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
| residue 1 | residue 2   | Upper Bound (Å) | Experimental Evidence | Reference |
|----------|-------------|-----------------|------------------------|-----------|
| 168      | 211-218     | ≤ 11.7          | mutagenesis 1          |           |
| 171      | 210         | ≤ 13.8          | MTS-3-MTS crosslink 2  |           |
| 171      | 474         | ≤ 11.0          | MTS-1-MTS crosslink 2  |           |
| 172      | 211-218     | ≤ 11.7          | mutagenesis 1          |           |
| 174      | 210         | ≤ 15.1          | MTS-4-MTS crosslink 1  |           |
| 176      | 369         | ≤ 7.1           | DTT activation 3       |           |
| 263      | 474         | ≤ 7.1           | Cu-Phe induced disulfide 4 | |
| 269      | 219         | ≤ 17.5          | MTS-6-MTS crosslink 2  |           |
| 270      | 219         | ≤ 15.1          | MTS-4-MTS crosslink 2  |           |
| 284      | 210         | ≤ 15.1          | MTS-4-MTS crosslink 2  |           |
| 473      | 210         | ≤ 12.5          | MTS-2-MTS crosslink 2  |           |
| 474      | 210         | ≤ 12.5          | MTS-2-MTS crosslink 2  |           |
| 475      | 210         | ≤ 15.1          | MTS-4-MTS crosslink 2  |           |
| 476      | 210         | ≤ 15.1          | MTS-4-MTS crosslink 2  |           |
| 477      | 210         | ≤ 15.1          | MTS-4-MTS crosslink 2  |           |

**Table 2. Experimentally derived constraints added to the ENaC α subunit model.** Additional constraints consisted of residues (Site 1) with a set maximum distance to another residue(s) (Site 2). Constraints were added to the alpha ENaC model based on the results of 1Kashlan et al, 2011 (10), 2Kashlan et al, 2012 (20), 3Kashlan et al. 2015 (24), and 4Figure 2.
ENaC finger–thumb domain interface cysteines

Figure 1

A

B

finger domain

238 rdnpgqdkcwk-gqll-sq-nx-gltyqssqsvqvre α ENaC
195 ssmehbheknk1-ygfflemdtsstcaytfsraganwga γ ENaC
181 k---k---kvamrlc-ea-gtvvlrlrntatqaye β ENaC
145 -----------------------------------------lnkpkpfnml α ASIC1a

αC256 αC263 γC213 γC220 (Cys-2) (Cys-3)

C

Top of the thumb domain

452 ml-qdrlyifytikpkqyvel-nkqssqkqcykllqanf α ENaC
409 mveetapayyssqipppasrnqcsasqcyqklycall γ ENaC
393 mrcr-qghlyplypeoysmnrydpayywcv1nnsmsv β ENaC
318 lsrvttrmvwh---mrpatympoqyk---la-dpaflα ASIC1a

γC415 (Cys-11) γC429 (Cys-12)
ENaC finger–thumb domain interface cysteines

Figure 2

A

Cu or Cu-Phe

Na 110 110 DTT Amil

wild type

1.0 μA

0.5 μA

30 s

0.5 μA

0.1 μA

αY474C

αK476C

B

Cu-Phe

Na 110 110 DTT Amil

αC263A,Y474C

1 μA

30 s

αC256A,Y474C

0.2 μA

C



\frac{I_{Cu-Phe}}{I_0}

WT

αY474C

αK476C

αC256A,Y474C

αC263A,Y474C

* NS **

1.00

0.75

0.50

0.25
Figure 3

A

MTS-2-MTS

| Na 110 | Na 110 | DTT | Amil |

wild type

1 µA

60s

γD115C

1 µA

B

\[ \frac{(I_{\text{MTS}} - I_{\text{MTS}})}{I_0} \]

\( MTS-\alpha-MTS: 1 2 3 \)

wild type γD115C
ENaC finger–thumb domain interface cysteines

Figure 4

A

Finger

Thumb

C210  C220

C115

C410  C440

B

MTS-2-MTS

- | | - | DTT amiloride

γD115C,C213A

0.1 μA

60 s

γD115C,C220A

γD115C,C415A

0.2 μA

γD115C,C429A

0.3 μA

γD115C,C440A

0.2 μA

C

\[ \frac{(I_{\text{max}} - I_0)}{I_0} \]

γD115C

**** *** * wild type

γD115C,C213A

γD115C,C220A

γD115C,C415A

γD115C,C429A

γD115C,C440A
Figure 5

A

- | MTS | - | DTT | Amil

γC213A

γC213A, C415A

γC213A, C429A

γC220A

γC220A, C415A

γC220A, C429A

Wild type

γC213A, C220A

B

\[ \frac{(MTS_{n} - MTS_{0})}{MTS_{0}} \]

- \( \gammaC213A \)

- \( \gammaC213A, C415A \)

- \( \gammaC213A, C429A \)

- \( \gammaC220A \)

- \( \gammaC220A, C415A \)

- \( \gammaC220A, C429A \)

- Wild type

- \( \gammaC213A, C220A \)
ENaC finger–thumb domain interface cysteines

Figure 7

A

Amiloride-sensitive current (nA)

cC56A, IY74C

cC63A, IY74C

c213A

B

Relative Surface Expression

c63A, IY74C

c213A

c220A

c415A

c429A
Figure 8

A

| 3 µA | 2 min |
|-------------|

B

C

Rapid Inhibition (%)

Sustained Inhibition (%)

wild type

αC256A

αC263A

γC415A

γC429A

amiloride
Figure 9

A

B

C

D

ENaC finger–thumb domain interface cysteines
Conserved cysteines in the finger domain of the epithelial Na+ channel α and γ subunits are proximal to the dynamic finger-thumb domain interface
Brandon M Blobner, Xue-Ping Wang and Ossama B Kashlan

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