Multiple epithelial Na\(^+\) channel domains participate in subunit assembly

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Bruns, James B., Baofeng Hu, Yoon J. Ahn, Shaohu Sheng, Rebecca P. Hughley, and Thomas R. Kleyman. Multiple epithelial Na\(^+\) channel domains participate in subunit assembly. Am J Physiol Renal Physiol 285:F600–F609, 2003.—Epithelial sodium channels (ENaCs) are composed of three structurally related subunits that form a tetrameric channel. The Xenopus laevis oocyte expression system was used to identify regions within the ENaC \(\alpha\)-subunit that confer a dominant negative phenotype on functional expression of \(\alpha\)-ENaC to define domains that have a role in subunit-subunit interactions. Coexpression of full-length mouse \(\alpha\)ENaC with either 1) the \(\alpha\)-subunit first membrane-spanning domain and short downstream hydrophobic domain (\(\alpha\)-M1H1); 2) \(\alpha\)-M1H1 and its downstream hydrophilic extracellular loop (\(\alpha\)-M1H1-ECL); 3) the membrane-spanning domain of a control type 2 transmembrane protein (glutamyl transpeptidase; \(\gamma\)-GT) fused to the \(\alpha\)-ECL (\(\gamma\)-GT-\(\alpha\)-ECL); 4) the extracellular domain of a control type 1 transmembrane protein (Tac) fused to the \(\alpha\)-subunit second membrane-spanning domain and short upstream hydrophobic domain (Tac-\(\alpha\)-H2M2); or 5) the \(\alpha\)-subunit cytoplasmic COOH terminus (\(\alpha\)-C\(t\)) significantly reduced amiloride-sensitive sodium currents in \(X\). laevis oocytes. Functional expression of Na\(^+\) channels was not inhibited when full-length \(\alpha\)ENaC was coexpressed with either 1) the \(\alpha\)-ECL lacking a signal-anchor sequence, 2) \(\alpha\)-M1H1 and \(\alpha\)-ENaC as a fusion protein, 3) full-length \(\gamma\)-GT, or 4) full-length Tac. Furthermore, the expression of ROMK channels was not inhibited when full-length ROMK was coexpressed with either \(\alpha\)-M1H1-ECL or Tac. Full-length FLAG-tagged \(\alpha\), \(\beta\), or \(\gamma\)-ENaC communoprecipitated with myc-tagged \(\alpha\)-M1H1-ECL, whereas wild-type \(\gamma\)-GT did not. These data suggest that multiple sites within the \(\alpha\)-subunit participate in subunit-subunit interactions that are required for proper assembly of the heterologomeric ENaC complex.

amiloride-sensitive sodium channel; dominant negative mutants

THE EPITHELIAL Na\(^+\) CHANNEL (ENaC) has a key role in the regulation of urinary Na\(^+\) reabsorption, extracellular fluid volume homeostasis, control of blood pressure, and airway fluid levels (14, 25, 57, 63). ENaCs are composed of three homologous subunits, termed \(\alpha\), \(\beta\), and \(\gamma\), that assemble into a tetrameric structure with a subunit stoichiometry of 2:1:1:1 (16, 34), although an alternative subunit stoichiometry has been proposed (64). Mutations leading to clinical disorders have been identified in all three subunits. Gain-of-function mutations in the \(\beta\)- and \(\gamma\)-subunits have been identified in patients with Liddle’s syndrome, a disorder characterized by volume expansion and hypertension (22, 26, 51, 59, 67), whereas ENaC loss-of-function mutations have been identified in patients with type I pseudohypaldosteronism, a disorder characterized by volume depletion, hypotension, and hyperkalemia (10, 58).

Mouse ENaC subunits are composed of between 638 and 699 amino acids and share a common topology (3). Each subunit has two membrane-spanning domains (M1 and M2) and adjacent extracellular hydrophobic domains (H1 and H2) separated by a large extracellular loop (ECL) (8), whereas the NH2 and COOH termini of each subunit are intracellular (7, 43, 55). The combined application of site-directed mutagenesis and oocyte expression has yielded important findings regarding the structure/function relationships of ENaC (5, 32, 44, 52). Two amiloride-binding sites have been identified within the ENaC subunits, one within the pore region of each subunit and a second within the ECL of the \(\alpha\)-subunit (27, 33, 46). The main selectivity filter has been localized to a three-residue tract ([G/S/X]S) within the H2 region (or equivalently termed as pre-M2 region) (29–31, 47, 56). Mutations that alter ENaC gating have been found in the NH2 termini (20, 21), ECL (50), pore regions (48, 53) and nearby regions (18), M2 domains (19), and COOH termini (12).

Limited information is available regarding the identification of domains within the heterologomeric Na\(^+\) channel complex that participate in subunit-subunit interactions and promote subunit oligomerization. Na\(^+\) channel subunits are thought to assemble in the endoplasmic reticulum (ER) where they undergo N-linked glycosylation (2, 7, 11, 13, 23, 40, 41, 45, 55, 65). The interactions of newly synthesized Na\(^+\) channel subunits within the ER must be of a sufficient affinity to promote Na\(^+\) channel assembly. A previous study by Adams and co-workers (2) showed that the NH2-termin-
nal domain of γ-ENaC interacted with and prevented functional expression of full-length αβγ-ENaC. Similarly, our previous work indicated that the NH2-terminal domain of α-ENaC interacted with full-length αβγ-ENaC and prevented the expression of amiloride-sensitive currents in *Xenopus laevis* oocytes (3), whereas results published by Chalfant and co-workers (9) showed that NH2 terminally deleted α-, β-, or γ-ENaC prevented functional expression of full-length αβγ-ENaC. We have extended these observations and have identified multiple domains throughout α-ENaC that, when coexpressed with full-length αβγ-ENaC, confer a dominant negative phenotype. These domains may have an important role in facilitating subunit-subunit interactions that allow for proper assembly and functional expression of the heterooligomeric Na+ channel.

**MATERIALS AND METHODS**

*Materials.* All chemicals were purchased from Sigma (St. Louis, MO) or as specified otherwise.

*Mouse ENaC constructs.* Mouse (m) α- and γ-ENaC cDNAs were previously cloned into pBluescript SK(–) (Stratagene, La Jolla, CA) (3). The following α-m-ENaC domains were generated by PCR: α-M1H1-ECL containing S102-S568; α-M1H1 containing S102-R164; α-M1H1-cytoplasmic COOH terminus (Ct) containing S102-R164 fused to H613-L699; α-Ct containing H613-L699; γ-glutamyl transpeptidase (GT)/ECL containing the short cytoplasmic tail and transmembrane domain of rat γ-GT (M1-T28) (28) fused to the α-subunit’s extracellular loop (Y165-S568); and Tac/M2H2 containing the extracellular domain of the human interlukin-2 receptor α-subunit (Tac, M1-Q240) (39) fused to the α-subunit’s second hydrophobic and transmembrane domains (V569-R618). COOH terminally truncated αβγ-ENaCs were generated by placing stop codons at H613, R564, and R583 of α-, β-, and γ-, respectively. The FLAG epitope was added to the COOH terminus of the α-, β-, and γ-cDNAs, the V5 epitope was added to the COOH terminus of the β-subunit and α-Ct cDNA, and the myc epitope was added to the COOH termini of α-M1H1-ECL and γ-GT-ECL cDNAs. All epitope-tagged constructs were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). All constructs were confirmed by automated DNA sequencing at sequencing facilities at the University of Pennsylvania or at the University of Pittsburgh. cRNAs were synthesized from the above constructs in linearized pBluescript or pcDNA3.1 using the T4 or T7 mMESSAGEm MAChINE kit (Ambion, Austin, TX). cRNAs were stored at −80°C and diluted in nuclease-free water before injection.

*Oocyte preparation and injection.* Oocytes were obtained from adult female *X. laevis* using protocols approved by Institutional Animal Care and Use Committees at the University of Pennsylvania and the University of Pittsburgh. Stage V and VI *X. laevis* oocytes were enzymatically defolliculated in 2 mg/ml type IV collagenase and then maintained at 18°C in modified Barth’s saline [MBS; (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO3, 15 HEPES, 0.3 Ca(NO3)2, 0.41 CaCl2, and 0.82 MgSO4, as well as 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamicin sulfate, pH 7.4]. Oocytes were microinjected with mouse α-, β-, and γ-ENaC cRNAs (2 ng/subunit) with or without test cRNAs (20 ng, unless otherwise indicated) in 50 nl nuclease-free H2O. Oocytes were also coinjected with αH613X, βR564X, and γR583X cRNAs (0.5 ng/subunit) with or without α-Ct cRNA (5 ng). Whole cell currents were measured 1–2 days after cRNA injections.

*Whole cell current measurements.* The two-electrode voltage-clamp technique was used to measure the amiloride-sensitive whole cell inward currents with a DigiData 1200 interface (Axon Instruments, Foster City, CA) and a TEV 200A Voltage Clamp Amplifier (Dagan, Minneapolis, MN). Data acquisition and analyses were performed using pClamp 7.0 software (Axon Instruments) with a Pentium II-based PC (Gateway 2000, N. Sioux City, SD). Recording pipettes were pulled from borosilicate glass capillaries (World Precision Microinstruments, Sarasota, FL) and backfilled with a 3 mol/l KCl solution (pH 7.2, 300 mOsm) containing 2.4 NaHCO3, 2.5 MgCl2, 100 TEA, 5 EGTA, 5 sucrose, and 10 HEPES. Currents were measured from oocytes maintained at 18°C in modified Barth’s saline (MBS) containing 87.6 NaCl, 1 KCl, 2.4 NaHCO3, 15 HEPES, 0.3 Ca(NO3)2, 0.41 CaCl2, and 0.82 MgSO4, as well as 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamicin sulfate, pH 7.4. Whole cell currents were measured 1–2 days after cRNA injections.

**Figs. 1, 2, and 3.** Topology of α-subunit of the epithelial Na+ channel (α-ENaC) and the α-ENaC domains and chimeras tested for the ability to confer a dominant negative phenotype. The topology of full-length α-mouse (m) ENaC is illustrated (top), followed by the α-ENaC domains and chimeras used in this study. M1 and M2, hydrophobic 1st and 2nd membrane-spanning domains; H1 and H2, largely hydrophobic regions immediately following M1 and immediately preceding M2, respectively. The short cytoplasmic and transmembrane domains of γ-glutamyl transpeptidase (γ-GT), a type II integral membrane protein, and the extracellular domain of the IL-2 receptor α-chain (Tac), a type I integral membrane protein, were fused to the α-subunit extracellular loop (α-ECL) and to α-H2M2, respectively, to ensure that α-ENaC domains were properly located in the lumen or membrane of the endoplasmic reticulum (ER).
Instruments, Sarasota, FL) and filled with 3 M KCl. Pipettes with tip resistances of 0.5–3 MΩ in a 110 mM NaCl bath solution were chosen for experiments. Oocytes were bathed in a solution containing (in mM) 96 NaCl, 2 KCl, 1.8CaCl₂, 1 MgCl₂, and 5 HEPES-NaOH, pH 7.4. All measurements were carried out at room temperature (22–25°C), and the bath solution was continuously perfused at 5–6 ml/min by gravity. Oocytes were typically incubated in the bath solution for at least 5 min before the current was recorded to allow currents to stabilize. Oocytes were clamped at –100 mV for 900 ms. Currents were measured at 600 ms after initiation of the clamp potential. Between recordings, the circuit was opened, allowing the oocytes to rest at the reversal potential. To minimize the contribution of variability in expression levels from different batches of oocytes, we measured the whole cell currents in oocytes co-injected with αβγ-mENaC cRNAs and in oocytes co-injected with αβγ-mENaC cRNAs plus an α-subunit construct cRNA, using oocytes obtained from a single batch. Oocytes from the two groups were clamped in an alternating manner so that the contribution of expression time to the measured currents was similar for each group. Measurements from repeated experiments, using oocytes from at least two frogs, were pooled to obtain a sufficient number of observations for statistical analysis.

Transient expression in Chinese hamster ovary cells. Wild-type Chinese hamster ovary (CHO) cells were cultured as previously described (4). Transient expression of mENaC subunits, dominant negative constructs, and/or γ-GT was carried out in CHO cells by infection with recombinant vaccinia (vT7CP) expressing T7 RNA polymerase, followed by transfection with expression vectors containing T7 promoters at the 5′-end of the cDNAs as previously described (42, 64). The full-length γ-GT used contained a substitution of asparagine for threonine at position 380. This mutation prevents the autocatalytic cleavage of γ-GT and eliminates enzymatic activity without affecting processing or trafficking (Hughey R, unpublished observations). vT7CP was a kind gift from Bernard Moss (National Institute of Allergy and Infectious Disease, Bethesda, MD). Transfections were carried out 30 min post-infection with lipofectamine reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). After overnight incubation, –1 × 10⁶ cells were solubilized at room temperature in 300 μl of a detergent solution (50 mM Tris·HCl, 1% Nonidet P-40, 0.4% deoxycholate, and 62.5 mM EDTA, pH 8.5) supplemented with 1% Protease Inhibitor Cocktail Set III (Calbiochem, La Jolla, CA). Insoluble material was removed by centrifugation in a microcentrifuge at 20,000 g for 7 min at 4°C. Supernatants were recovered and incubated overnight at 4°C after the addition of protein G-Sepharose (Sigma) and mouse monoclonal antibodies against either Flag (Anti-Flag M2, Sigma) or Myc (9E10, Santa Cruz Biotechnology, Santa Cruz, CA). The vitellin membrane was nonfat dry milk. Subsequently, the blot was incubated in PBS containing 1% nonfat dry milk and the indicated mouse monoclonal antibody at room temperature for 3 h. All blots were then washed extensively with PBS followed by a 45-min incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD) in PBS containing 1% nonfat dry milk. After an extensive washing in PBS, HRP bound to proteins was detected using Western Lightening Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA) and BioMax MR film (Eastman Kodak, Rochester, NY) as directed by the manufacturer.

mENaC surface expression in X laevis oocytes. Full-length αβγ-mENaC was coexpressed with or without α-M1H1-ECL in X laevis oocytes. The β-subunit was tagged with the V5 epitope at its COOH terminus. The vitellin membrane was manually removed with fine forceps and, after being washed...
ENaC DOMINANT NEGATIVE DOMAINS

F603

Fig. 3. α-M1H1-ECL and γ-GT-ECL inhibit functional ENaC expression. A: oocytes were coinjected with wild-type αβγ-mENaC cRNAs (2 ng/subunit) with or without a 10-fold excess (20 ng) of α-M1H1-ECL cRNA. Whole cell amiloride-sensitive Na+ currents were recorded 24–48 h after injection at a clamp potential of –100 mV (n = 60). *P < 0.001. B: oocytes were coinjected with wild-type αβγ-mENaC cRNAs (2 ng/subunit) with or without a 10-fold excess (20 ng) of γ-GT-ECL cRNA. Whole cell amiloride-sensitive Na+ currents were recorded as in A (n = 49–53). *P < 0.001. C: oocytes were coinjected with wild-type αβγ-mENaC cRNAs (2 ng/subunit) with or without a 10-fold excess (20 ng) of α-ECL cRNA (lacking a signal-anchor sequence). Whole cell amiloride-sensitive Na+ currents were recorded as in A (n = 22–25; P = NS). D: oocytes were coinjected with wild-type αβγ-mENaC cRNAs (2 ng/subunit) with or without a 10-fold excess (20 ng) of γ-GT cRNA, a type II integral membrane protein. Whole cell amiloride-sensitive Na+ currents were recorded as in A (n = 39; P = NS).

in MBS, the oocytes were incubated twice for 20 min with 1.5 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in MBS containing 10 mM triethanolamine, pH 9.0, on ice. Free biotin reagent was quenched by four 5-min washes in MBS containing 5 mM glycine. After three washes with MBS, 15 oocytes were solubilized on ice in 300 μl of a detergent solution (25 mM MES, pH 6.4, 200 mM NaCl, 1% Triton X-100, 60 mM n-octyl glucoside, 0.1% SDS, 0.5% Nonidet P-40, 0.02% sodium deoxycholate, 1% digitonin, 0.5% Tween 20, 0.02% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 2 mM Empigen BB). Insoluble material was removed by centrifugation for 15 min at 15,000 g at 4°C. Supernatants were recovered and incubated overnight at 4°C with immobilized streptavidin (Pierce). Streptavidin precipitates were then washed and subjected to SDS-PAGE and immunoblot analyses as above. Anti-V5 antibody (Invitrogen) was used to detect the ENaC β-subunit.

Statistical analysis. Data are expressed as means ± SE. Unpaired Student’s t-tests were used to assess significance. P < 0.05 was considered significant.

RESULTS

The X. laevis oocyte expression system was used to identify regions within the α-subunit that confer a dominant negative phenotype on αβγ-ENaC expression. α-ENaC topology and the α-ENaC constructs used for these studies are illustrated in Fig. 1. We previously observed that coexpression of full-length mouse αβγ-ENaC with the cytoplasmic NH2 terminus of the α-subunit inhibited functional Na+ channel expression based on amiloride-sensitive currents in X. laevis oocytes (3). Similarly, in this study we observed that coexpression of αβγ-ENaC with α-M1H1 also led to an inhibition of functional channel expression (Fig. 2A). This functional inhibition was dose dependant (Fig. 2B), suggesting that α-M1H1 was competing with full-length subunits for binding sites that promote Na+ channel assembly. Although α-M1H1 inhibited functional ENaC expression, expression of a fusion protein of α-M1H1 and α-M1H1-Ct did not inhibit functional ENaC expression (Fig. 2C), suggesting that the presence of the COOH terminus within the ER lumen may prevent or limit interactions between α-M1H1-Ct and full-length subunits.

We observed that coexpression of αβγ-ENaC with α-M1H1-ECL also inhibited functional Na+ channel expression (Fig. 3A). Further experiments were carried out to determine whether α-ECL, independently of α-M1H1, had a role in conferring a dominant negative phenotype. To ensure proper orientation of the ECL within the ER lumen, we generated a fusion protein composed of the membrane-spanning domain of a control type 2 integral membrane protein, γ-GT, fused to α-ECL (γ-GT-ECL). Coexpression of αβγ-ENaC with γ-GT-ECL resulted in an inhibition of functional Na+ channel expression (Fig. 3B). In contrast, coexpression

Fig. 4. α-M1H1 and the short cytoplasmic tail and transmembrane domain of γ-GT are functional signal-anchor sequences allowing for ER translocation of α-ECL. myc-Tagged α-M1H1-ECL or γ-GT-ECL cDNAs were transiently expressed in Chinese hamster ovary (CHO) cells as described (see MATERIALS AND METHODS). After overnight incubation, cells were solubilized and the expressed proteins were immunoprecipitated with an anti-myc antibody (9E10). Immunoprecipitates were treated with or without N-glycosidase (PNGase) F, followed by SDS-PAGE and immunoblot analysis. Both constructs showed a significant shift in apparent molecular weight after PNGase F treatment.
of αβγ-ENaC with either the α-ECL (lacking a signal-anchor sequence) or full-length γ-GT did not inhibit functional channel expression (Fig. 3, C and D).

α-ECL has six consensus sites for N-linked glycosylation (7). To confirm that the ECL within the α-M1H1-ECL or γ-GT-ECL constructs was properly transferred into the ER lumen, we transiently expressed myc epitope-tagged α-M1H1-ECL or γ-GT-ECL in CHO cells. The expressed constructs were immunoprecipitated from cell lysates, and N-glycans were cleaved by treatment with PNGase F. Both α-M1H1-ECL and γ-GT-ECL displayed a shift to a lower apparent molecular weight after treatment with PNGase F (Fig. 4), confirming the addition of N-glycans to these polypeptides and indicating that the ECLs within these constructs were present within the ER lumen.

Previous studies have shown that residues within M2 and H2 form the pore of the channel (29–31, 36, 37, 47–49). We examined whether α-H2M2 conferred a dominant negative phenotype. The NH2-terminal ectodomain of a control type 1 integral membrane protein, the human interleukin-2 receptor α-subunit (commonly referred to as Tac), was fused to α-H2M2 (Tac-α-H2M2) to ensure proper orientation of α-H2M2 in the ER membrane. Coexpression of αβγ-ENaC with Tac-α-H2M2 inhibited functional Na+ channel expression (Fig. 5A), whereas full-length Tac did not confer a dominant negative phenotype (Fig. 5B).

Fig. 5. Tac-α-H2M2 inhibits functional ENaC expression. A: oocytes were coinjected with wild-type αβγ-mENaC cRNAs (2 ng/subunit) with or without a 10-fold excess (20 ng) of Tac-α-H2M2 cRNA. Whole cell amiloride-sensitive Na+ currents were recorded 24–48 h after injection at a clamp potential of −100 mV (n = 24). *P < 0.001. B: oocytes were coinjected with wild-type αβγ-mENaC cRNAs (2 ng/subunit) with or without a 10-fold excess (20 ng) of full-length Tac cRNA. Whole cell amiloride-sensitive Na+ currents were recorded as in A (n = 20; P = NS).

Fig. 6. α-COOH terminus inhibits functional expression of full-length, but not COOH terminally truncated, αβγ-ENaC. A: oocytes were coinjected with wild-type αβγ-mENaC cRNAs (2 ng/subunit) with or without a 10-fold excess (20 ng) of α-COOH terminal (Ct) cRNA. Whole cell amiloride-sensitive Na+ currents were recorded 24–48 h after injection at a clamp potential of −100 mV (n = 24). *P < 0.001. B: oocytes were coinjected with COOH terminally truncated αβγ-mENaC (ENaCΔC; α-H613X, β-R564X, γ-R583X) cRNAs (0.5 ng/subunit) with or without a 10-fold excess (5 ng) of α-Ct cRNA (n = 20; P = NS). C: oocytes were coinjected with wild-type ROMK cRNA (2 ng) with or without a 10-fold excess (20 ng) of α-M1H1 or α-Ct cRNA. Whole cell K+ currents were recorded 24–48 h after injection using the 2-electrode voltage-clamp technique at a clamp potential of −100 mV in the absence or presence of 5 mM BaCl2. For these experiments, bath NaCl was replaced with KCl. Ba2+-sensitive K+ currents were unchanged in oocytes coinjected with either α-M1H1 or α-Ct (n = 15–16; P = NS).
We also observed that coexpression of α-Ct with full-length αβγ-ENaC inhibited the expression of functional Na⁺ channels (Fig. 6A). In contrast, coexpression of ENaC subunits lacking their COOH termini with α-Ct did not alter functional Na⁺ channel expression (Fig. 6B), suggesting that direct interactions exist between ENaC intracellular domains. As simultaneous overexpression of several proteins may overload the translation machinery of a cell, leading to a false dominant negative phenotype, we performed similar experiments with an unrelated channel, ROMK. The expression of ROMK channels was not inhibited when full-length ROMK was coexpressed with either α-Ct or α-M1H1 (Fig. 6C), suggesting that multiple domains within the α-subunit confer a dominant negative phenotype, presumably by specifically associating with full-length ENaC subunits and preventing the assembly of tetrameric channels composed of full-length subunits.

Experiments were performed to examine whether α-M1H1-ECL binds to full-length ENaC subunits with sufficient affinity to allow for coimmunoprecipitation. α-M1H1-ECL with a COOH-terminal myc tag was transiently coexpressed with full-length, COOH-terminal FLAG-tagged α-, β-, or γ-ENaC, or full-length γ-GT (control) in CHO cells. Coimmunoprecipitation was observed when full-length ENaC subunits were immunoprecipitated, and subsequent immunoblots were probed for α-M1H1-ECL (Fig. 7A). We also observed coimmunoprecipitation when α-M1H1-ECL was immunoprecipitated, and subsequent immunoblots were probed for full-length ENaC subunits (Fig. 7B). α-M1H1-ECL did not coimmunoprecipitate with full-length γ-GT (Fig. 7A). We examined whether the dominant negative phenotype observed with coexpression of α-M1H1-ECL was associated with an inhibition of surface expression of Na⁺ channels. Full-length αβγ-ENaC were coexpressed with or without α-M1H1-ECL in X. laevis oocytes. The β-subunit was tagged with the V5 epitope at its COOH terminus. The vitellin membrane was removed, and surface proteins were labeled by treatment with membrane-impermeant sulfo-NHS-SS-biotin. Oocytes were homogenized/solubilized, and biotinylated (i.e., cell surface) proteins were precipitated with streptavidin-agarose. Precipitated proteins were subjected to SDS-PAGE and immunoblot analy-
sues to detect the ENaC β-subunit using anti-V5 antibody. Coexpression of αβγ-ENaC and α-M1H1-ECL in *X. laevis* oocytes was associated with a marked reduction in surface expression of β-ENaC (Fig. 8). We also examined whole cell expression of selected dominant negative constructs in oocytes coexpressed with αβγ-ENaC cRNAs and either α-M1H1-ECL, γ-GT-ECL, or α-Ct cRNA to confirm that these constructs were expressed in oocytes. Figure 9 illustrates that α-M1H1-ECL, γ-GT-ECL, and α-Ct were readily detected by immunoprecipitation followed by immunoblotting.

**DISCUSSION**

The interactions of newly synthesized ENaC subunits within the ER must be of sufficient affinity to promote Na+ channel assembly. As thousands of polypeptides are being synthesized on the ribosomes of a cell at any given time (68), it is likely that there are multiple sites of interaction between channel subunits that help to drive the assembly of this multimeric protein by increasing the affinity of intersubunit associations. Previous studies have shown that polyclonal interactions may increase binding affinity by orders of magnitude compared with monovalent interactions. For example, polyclonal immunoglobulins can bind polyclonal antigens with “apparent” affinity 1,000-fold higher than that of a monovalent immunoglobulin species (24). This increase in apparent affinity was attributed to a greatly reduced rate of dissociation and not to a change in the intrinsic affinity of the antibody.

Our work indicates that multiple α-subunit domains, when coexpressed with full-length αβγ-ENaC, confer a dominant negative phenotype. These domains include 1) the cytoplasmic NH2 terminus (3); 2) α-M1H1; 3) α-M1H1-ECL; 4) the ECL itself when fused to a type 2 signal-anchor sequence to facilitate ER translocation; 5) α-H2M2, when properly oriented in the ER membrane by fusion to a type 1 start transfer sequence; and 6) α-Ct. Furthermore, the cellular localization of an ENaC domain (cytoplasm or ER lumen) was an important factor in determining whether it conferred a dominant negative phenotype. For example, the ECL only conferred a dominant negative phenotype when translated into the ER lumen by fusion to a signal-anchor sequence (α-M1H1 or γ-GT). Similarly, the COOH terminus conferred a dominant negative phenotype when expressed in the cytoplasm but did not inhibit functional expression when translated into the ER lumen by fusion to a signal-anchor sequence (α-M1H1). The mislocalization of the COOH terminus within the α-M1H1-Ct construct might hinder interactions between α-M1H1 with full-length ENaC subunits, which could prevent M1H1 from inhibiting functional ENaC expression. Alternatively, exposure of the normally cytoplasmic COOH terminus to the lumen of the ER may have induced the rapid degradation of the α-M1H1-Ct before α-M1H1 could interact with full-length subunits.

Interactions of any of the dominant negative constructs with full-length α-, β-, or γ-ENaC could inhibit functional ENaC expression by causing the sequestration of full-length subunits in the ER, by interfering with the function of channels at the plasma membrane, or by inducing the ER-associated degradation of full-length channel subunits associated with the dominant negative constructs in nonfunctional channel complexes. Dominant negative-induced degradation of full-length channel subunits was observed in the suppression of α-ENaC currents mediated by the overexpression of truncated γ-subunits (2) and also in the suppression of Kir 4.1 currents by the coexpression of members of the Kir 3.0 family (61). We observed that the α-M1H1-ECL dominant negative construct was
associated with a marked reduction in surface expression of the full-length β-subunit, when coexpressed with full-length α- and γ-subunits, suggesting that overexpression of the α-M1H1-ECL construct led to the sequestration and/or degradation of full-length channel subunits in the ER; however, other possible explanations such as enhanced degradation in post-ER compartments cannot be excluded.

These results, together with previous reports (2, 3, 9), suggest the presence of multiple sites of intersubunit association within the heterooligomeric Na\(^+\) channel complex and are in agreement with studies demonstrating that other oligomeric ion channels have multiple sites of intersubunit interactions. For example, Tu et al. (60) used a dominant negative approach and demonstrated that multiple sites within the central core of Kv1.3 were involved in intersubunit association. Similarly, multiple sites of interaction have been demonstrated in the homooligomerization of Kir 1.1a channels (35) and in the heterooligomerization of Kir3.1 and Kir3.2 (66). These domains included the NH\(_2\)-terminal, core (i.e., M1 through M2), and COOH-terminal domains.

The ECL of each ENaC subunit contains 16 conserved cysteine residues, suggesting a complex organization of the loop structures. Mutations of the first, sixth, eleventh, and twelfth cysteine residues have been shown to lead to reduced surface expression and channel activity of rat αβγ-ENaC in oocytes (17). In addition, whole or partial deletion of the α-ECL resulted in no functional expression of αβγ-ENaC in oocytes (Sheng S and Kleyman TR, unpublished observations), and it was reported that even a six-residue deletion within the α-ECL led to no detectable current or surface protein expression (31). These observations are consistent with an expectation that the ECL has a role in ENaC assembly. Similarly, it is expected that α-M1H1 and α-H2M2 would have a role in subunit assembly, as the assembly of multiple transmembrane domains requires helix-helix interactions to ensure proper packing (1, 15, 62). However, the role of the COOH terminus in ENaC assembly is less clear, as truncation of the entire COOH terminus of the β- or γ-subunit in fact increases channel activity and cell surface density (52). While the α-Ct confers a dominant negative phenotype on functional ENaC expression, it should be noted that a domain that confers a dominant negative phenotype may be involved in, but not required for, subunit assembly. It is possible that the mechanism of inhibition of functional ENaC expression by α-Ct may differ from that observed with the other constructs. For example, α-Ct may confer a dominant negative phenotype by preventing interactions with accessory proteins that facilitate functional channel expression.

Truncations of ENaC subunits have been described in patients with autosomal recessive pseudohypoaldosteronism type I (6, 10). Bonny et al. (6) reported that coexpression of an α-subunit truncated just before the pore region with full-length β and γ allowed for functional ENaC expression in X. laevis oocytes. However, the onset of expression was delayed 48 h and the magnitude of the expressed current was markedly reduced with respect to wild-type αβγ-ENaC. The truncated α-subunit was not expressed with full-length αβγ-ENaC to test for a dominant negative effect. It is tempting to speculate that heterozygotes expressing both truncated and full-length α-ENaC subunits may have reduced levels of Na\(^+\) channel expression due to a dominant negative effect on channel assembly.

Transgenic overexpression of dominant negatives has become a useful tool for the in vivo study of ion channel function. For example, London and colleagues (38) have used the overexpression of a Kv1.1 dominant negative construct to disrupt Shaker-like K\(^+\) channel function in the hearts of transgenic mice. Beyond providing insight into α-ENaC domains that may be important in Na\(^+\) channel assembly, these data may prove useful for a dominant negative knockout of ENaC function in cell lines and animals.

In summary, our results indicate that multiple domains throughout α-ENaC, when coexpressed with αβγ-ENaC, confer a dominant negative phenotype. We propose that these domains may play an important role in facilitating and stabilizing subunit-subunit interactions that allow for the proper assembly and functional expression of the heterooligomeric ENaC.

DISCLOSURES

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