Activation of a latent nuclear localization signal in the NH$_2$ terminus of $\gamma$-ENaC initiates feedback regulation of channel activity

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Mironova E, Stockand JD. Activation of a latent nuclear localization signal in the NH$_2$ terminus of $\gamma$-ENaC initiates feedback regulation of channel activity. Am J Physiol Renal Physiol 298: F1188–F1196, 2010. First published February 10, 2010; doi:10.1152/ajprenal.00600.2009.—Proteolytic enzymes cleave the epithelial Na$^+$ channel (ENaC) at several positions releasing, in part, the NH$_2$ terminus of the $\gamma$-subunit. Cleavage increases ENaC activity by increasing open probability; however, the role of polypeptides cleaved from the channel core remains unclear. We find that the cytosolic NH$_2$ terminus of $\gamma$-ENaC unexpectedly targets to the nucleus being particularly strong in nucleoli. In contrast, the cytosolic COOH terminus targets to the cytoplasm and plasma membrane in a manner similar to full-length subunits. Targeting of the cytosolic NH$_2$ terminus of $\gamma$-ENaC to the nucleus has functional consequences for coexpression of eGFP-fusion proteins containing this segment of the channel, but not the COOH terminus, decrease ENaC activity in a dose-dependent manner. The mechanism of this negative regulation is associated with a decrease in the functional half-life of ENaC at the plasma membrane. Inspection of the primary amino acid sequence of $\gamma$-ENaC reveals possible nuclear localization signals (NLS) conserved at the extreme NH$_2$ terminus and just preceding the first transmembrane domain. Disruption of the putative NLS preceding the first transmembrane domain in $\gamma$-ENaC but not that at the extreme NH$_2$ terminus abolishes both targeting to the nucleus and negative regulation of ENaC activity. These findings are consistent with the release of the NH$_2$ terminus of $\gamma$-ENaC following cleavage being functionally important for signaling to the nucleus in a manner similar to Notch signaling and release of the cytosolic COOH-terminal tail of polycystin-1.

hypertension; aldosterone; polycystin-1; Notch

THE EPITHELIAL Na$^+$ CHANNEL (ENaC) is a noninactivating, voltage-independent, Na$^+$–selective ion channel (22, 32). This channel is a member of the ENaC/degenerin ion channel superfamily (2, 12, 32). Channel subunits in this superfamily share a common tertiary structure, having short intracellular NH$_2$ and COOH termini and a large extracellular region bound by two membrane-spanning domains with the bulk of the channel protein being extracellular (23, 31, 46). ENaC is an obligatory heterotrimer formed of similar but distinct $\alpha$-, $\beta$-, and $\gamma$-subunits (12, 32, 47).

Channels in the ENaC/degenerin superfamily have a wide tissue distribution, including being in neuronal and epithelial tissues (4, 32). Because of this, and their ability to carry depolarizing inward Na$^+$ currents in response to diverse extracellular and intracellular cues, family members serve important physiological roles. For instance, the activity of ENaC is limiting for vectorial Na$^+$ transport across many epithelial barriers (22).

Consequently, ENaC plays a central role in setting mucus hydration and fluidity, as well as systemic Na$^+$ and water balance to influence blood pressure. As such, ENaC is a key target for hormones involved in feedback regulation of blood pressure and setting water reabsorption in air spaces. Indeed, loss of ENaC function causes fluid accumulation in the lungs and renal salt and water wasting; in contrast, gain of ENaC function is causative for inappropriate renal salt conservation and associated hypertension (7, 30, 35, 44).

As ENaC matures, intracellular and extracellular proteases cleave the channel in a manner akin to posttranslational processing of many other membrane-spanning proteins (33). Specifically, both the $\alpha$- and $\gamma$-subunits of ENaC are cleaved (27–29, 37). It currently is not clear whether ENaC cleavage is constitutive or dynamic, capable of being regulated by signaling, although contemporary thinking is that it is dynamic but irreversible, with differential cleavage possibly being responsible, in part, for the wide diversity in open probabilities observed for ENaC at the plasma membrane (33). What is clear is that cleavage releases polypeptides from the channel core to increase activity by increasing channel open probability. Cleavage sites for the trans-Golgi proprotein convertase serine protease furin, and the extracellular glycosylphosphatidylinositol-anchored channel-activating proteases CAP1 (also called prostasin) and CAP2 (also called TMPRSS4), as well as plasmin, elastase, and kallikrein, have been identified in the extracellular region of the $\gamma$-subunit just after the first transmembrane (1, 11, 15, 16, 19, 21, 27, 40–42, 50). As defined in the crystal structure of cASIC recently provided by Jasti and colleagues (23, 31), this region of $\gamma$-ENaC is in the finger domain, which is a hypervariable area of ENaC/degenerin proteins (49). While cleavage increases open probability, the mechanism for this currently is obscure. Moreover, the fate and function of polypeptides cleaved from the channel core also remain unclear. Evidence has been provided that cleavage releases inhibitory domains, permitting an increase in channel open probability (27, 33). A variation of this theme is that cleavage results in the physical separation of the NH$_2$ terminus of $\alpha$-ENaC from the channel core, allowing channel relaxation into a conformation conducive to increases in open probability (26). Interestingly, in this latter study, the NH$_2$ terminus of $\alpha$-ENaC, including its first transmembrane domain, had a different fate compared with the remaining channel core with respect to membrane residency and retrieval. This gives rise to the possibility that polypeptides released from the core of ENaC following cleavage may serve signaling functions in addition to allowing the channel to adopt a configuration commensurate with higher open probability.

Precedence exists for such a possibility. The Notch signaling pathway, important for cell-cell communication, comes to mind (18, 20, 45). Signaling to the nucleus in this pathway...
arises from activation and subsequent ordered cleavage of the liganded Notch receptor, with induction leading to sequential cleavage first by extracellular proteases and then intracellular proteases to release the intracellular domain of the receptor (6, 18, 20, 34). The released fragment contains a nuclear localization signal (NLS) that targets it to the nucleus, particularly the nucleolus, where it then affects gene expression (18, 20).

Polycystin-1 (PC-1) is an integral membrane protein that serves as an anchor and chaperone protein promoting translocation of polycystin-2 (PC-2) to the plasma membrane (5, 24). Once at the membrane, PC-2, likely in association with PC-1 and possibly other TRP channel proteins, functions as a non-selective Ca\(^{2+}\)-permeable cation channel (3, 24). In addition to functioning as an ion channel, the PC-1/PC-2 complex also is capable of initiating/regulating cell signaling (5, 13, 36). PC-1 employs a mechanism similar to Notch receptors to signal to the nucleus. PC-1 is processed by proteolytic cleavage with induced cleavage, physically releasing a portion of the protein, the cytoplasmic COOH-terminal tail, to the nucleus to influence gene expression (5, 13, 36).

Because of the examples discussed above, the fact that the NH\(_2\) terminus of γ-ENaC, which contains sequences conforming to known NLSs (10, 14, 38), is cleaved from the channel core and that polypeptides cleaved from ENaC can, in some instances, have distinct fates from the channel core (26), we hypothesized that the NH\(_2\) terminus of γ-ENaC may have a PC-1 and Notch-like signaling function. To begin testing this idea, we asked two key questions: 1) what is the cellular localization of the NH\(_2\) terminus of γ-ENaC when released from the channel core; and 2) is there any proof that accumulation of the NH\(_2\) terminus of γ-ENaC in the nucleus affects cellular function/activity. We find that the NH\(_2\) terminus of γ-ENaC targets to the nucleus, where it then is capable of modulating activity of ENaC at the plasma membrane. The mechanism of this regulation involves decreases in the functional half-life of ENaC in the membrane and is dependent on the NH\(_2\) terminus of γ-ENaC targeting to the nucleus. A monopartite NLS located in the cytosolic region just preceding the first transmembrane domain is critical to targeting the NH\(_2\) terminus of γ-ENaC to the nucleus and dependent, negative regulation of ENaC within the plasma membrane.

METHODS

Chemicals, cells, and cDNA constructs. All chemicals were from Sigma (St. Louis, MO), Fisher (Pittsburgh, PA), or Calbiochem (Gibbstown, NY) unless noted otherwise. Plasmids encoding eCFP (CFP = cyan fluorescent protein) and eYFP (YFP = yellow fluorescent protein) targeted to the plasma membrane (eCFP-M/eYFP-M) and DsRed targeted to the nucleus were from Clontech. The mammalian expression vector encoding Nav1.5 was a gift from Dr. R. S. Kass. The mammalian expression vectors encoding α-, β-, and γ-mouse ENaC subunits with an NH\(_{2}\)-terminal myc-epitope tag or as NH\(_{2}\)-terminal eGFP/eCFP/eYFP-fusion proteins have been described previously (43, 47). As noted in these earlier publications, channels composed of epitope-tagged subunits or subunits as fusion proteins have indistinguishable function from channels composed of subunits lacking such modifications. The eGFP/eCFP/eYFP-fusion proteins (GFP = green fluorescent protein) containing either the cytosolic NH\(_{2}\) or COOH terminus of γ-mENaC have also been described previously (9, 25). Mutants of these latter fusion proteins were created in our laboratory with QuickChange (Stratagene) mutagenesis per the manufacturer’s instructions or outsourced to TOP Gene Technologies (Montreal, QC, Canada). Regardless of the source, every mutant was sequenced to ensure proper incorporation of the expected mutation and to confirm sequence identity, orientation, and reading frame.

Chinese hamster ovary (CHO) cells were from American Type Culture Collection. These cells were maintained with standard culture conditions (DMEM + 10% FBS, 37°C, 5% CO\(_{2}\)) as described previously (43, 47, 48). Recombinant mENaC was overexpressed in CHO cells by transfecting the appropriate expression plasmids using the Polyfect reagent (Qiagen, Valencia, CA) as described previously (17, 43). Electrophysiological and imaging experiments were performed 48–72 h after transfection.

The murine collecting duct principal cell line mpkCCD\(_{14}\) was a gift from A. Vandewalle. These cells have been described previously and were used and maintained in the current study under standard culture conditions (43). Electroporation was used to introduce expression plasmids into mpkCCD\(_{14}\) cells. In brief, mpkCCD\(_{14}\) cells were transfected using a Nucleofector Kit (Amaxa, Koln, Germany) and the Amaxa Nucleofector electroporation device following the manufacturer’s instructions (using the K-29 program). A total of 5 μg of plasmid DNA was introduced. As above, imaging experiments were performed 48–72 h after transfection.

Imaging. Fluorescence emissions from membrane markers and fluorophore-tagged ENaC were collected with confocal or epifluorescence imaging in live cells. All imaging experiments were performed using standard procedures in the Department of Physiology at the University of Texas Health Science Center San Antonio, Confocal Electrophysiology Core Facility (http://physiology.uthscsa.edu/new/core_facilities/confocal_patch.asp). The confocal system is built around an inverted Nikon Eclipse TE2000 microscope (Nikon Instruments, Melville, NY). In brief, confocal images were collected with ×40 (1.3 NA), ×60 (1.3 NA), and ×100 (1.49 NA) oil-immersion objectives and a high-speed, low-light-level (512 × 512 chip) Cascade 2 Photometric CCD camera (Roper Scientific, Tucson, AZ) interfaced with a PC running NIS-element AR fluorescence imaging software (Nikon Instruments). The fluorophores, eCFP, and eYFP, and eGFP, were excited with laser light from a 440-nm Melles Griot dual-pulsed solid-state diode laser, and 488- and 514-nm lines from an Argon-ion gas laser, respectively. Emissions from eGFP and eCFP/eYFP were collected through a 495-nm long-pass dichroic mirror complemented with a 500-nm long-pass emission filter and a 465/505-nm dual dichroic mirror complemented with a 480/555-nm dual band-pass emission filter set, respectively. For epifluorescence imaging, a ×40 objective (1.4 NA) on a system built around a Zeiss Axiosvert 200M inverted microscope was used. A mercury lamp fitted with 480/20- and 545/15-nm excitation filters was used to excite fluorophores. Emissions passed through 535/25- and 620/30-nm band-pass filter sets with images analyzed using Slidebook 4.2 software (3I, Denver, CO).

Electrophysiology. Whole-cell macroscopic current recordings of recombinant mENaC expressed in CHO cells were made under voltage-clamp conditions using standard methods (43, 48). Current through ENaC was the inward, amiloride-sensitive Na\(^{+}\) current at −80 mV with an extracellular bath solution of (in mM) 160 NaCl, 1 CaCl\(_2\), 2 MgCl\(_2\), and 10 HEPES (pH 7.4), and an intracellular pipette solution of (in mM) 120 CsCl, 5 NaCl, 2 MgCl\(_2\), 5 EGTA, 10 HEPES (pH 7.4), 2.0 ATP, and 0.1 GTP. Currents through ENaC were elicited with a voltage ramp (500 ms) from 60 to −100 mV forming a holding potential of 40 mV. Current recordings were acquired with an Axopatch 200B (Axon Instruments, Union City, CA) interfaced via a Digidata 1322A (Axon Instruments) to a PC running the pClamp 9.2 suite (Axon Instruments). All currents were filtered at 1 kHz. Whole-cell capacitance was routinely compensated and was approximately ∼9 pF for CHO cells. Series resistances, on average 2–5 MΩ, were also compensated.
Statistics. Summarized data are presented as means ± SE. Unpaired data were compared using a t-test or a one-way ANOVA with an appropriate posttest. *P < 0.05 is considered significant.

RESULTS

The cytosolic NH₂ terminus of γ-ENaC contains two clusters of conserved positively charged residues: one at the front end of the molecule and one just preceding the first transmembrane domain. These tracks of basic amino acids are similar to a monopartite NLS (10, 14, 38). Results in Fig. 1 demonstrate that the cytosolic NH₂ terminus of γ-mEnaC contains a latent NLS that can become functional. Shown in Fig. 1A are representative confocal fluorescence micrographs of a CHO cell expressing the membrane marker CFP-M (pseudocolored green; left), and a fusion protein containing the entire cytosolic NH₂-terminal portion of γ-mEnaC fused to eYFP (pseudocolored red; middle). The right panel shows a merged image. The white line across the middle of this cell indicates the region scanned in the line-scan presented in Fig. 1B. The line-scan of emissions emitted from eCFP (green) and eYFP (red) from the fluorophores in the cell in A. 

γ-mEnaC directs eYFP to the nucleus. The normalized line scan, shown in Fig. 1B for eCFP (green) and eYFP (red) emissions from Fig. 1A, reinforces this observation. In every instance, the cytosolic NH₂-terminal portion of γ-mEnaC directed the genetically conjugated fluorophore, be it eCFP, eYFP, or eGFP, to the nucleus, particularly to nucleoli (see also Fig. 2). In contrast, the cytosolic COOH-terminal portion of γ-mEnaC (γC; aa. 568-end), as shown in Fig. 2, does not localize to the nucleus but rather targets to the cytoplasm and plasma membrane in a manner similar to full-length ENaC subunits. [Unfused eCFP, eYFP, and eGFP are also cytosolic proteins that routinely localize to the cytoplasm in the absence of modification; not shown (25).]

Similar to that observed when expressing the fluorophore-tagged NH₂ terminus of γ-mEnaC alone in CHO cells, expressing (NH₂-terminal) eYFP-tagged full-length γ-mEnaC in murine mpkCCDc14 principal cells targeted the fluorophore, in

Fig. 2. Cytosolic NH₂-terminal portion of γ-mEnaC but not the cytosolic COOH terminus of this subunit directs conjugated fluorophores to the nucleus. Representative confocal fluorescence micrographs of CHO cells expressing eYFP-tagged (pseudocolored green) full-length ENaC subunits (top and bottom left) and either the cytosolic NH₂ terminus (top middle) or cytosolic COOH terminus (bottom middle) of γ-mEnaC as eCFP-fusion proteins (pseudocolored red) are shown. On the right top and bottom are merged images. The white scaling bar = 10 µm. Shown at bottom are sequences for the cytosolic NH₂ and COOH termini of γ-mEnaC used to create the respective fusion proteins.
some instances, to the nucleus. In contrast to CHO cells, which do not express all three endogenous ENaC subunits and likely do not express the full complement of proteolytic enzymes responsible for processing ENaC, renal epithelial mpkCCDc14 cells do. Appearance of eYFP in the nucleus from tagged full-length \( \gamma \)-mENaC was not an all-or-nothing event in mpkCCDc14 cells, compared with mpkCCDc14 (see fluorescence micrographs in the bottom row of Fig. 3) or CHO cells expressing \( \gamma N \) alone, but rather followed a gradient. Figure 3 shows two representative cases of eYFP localization in mpkCCDc14 cells when expressed as an eYFP-\( \gamma \)-mENaC fusion protein. The fluorescence micrographs in the top row of Fig. 3 show diffuse, cell-wide emissions, including emissions from the nucleus, from eYFP when conjugated (at least initially) to full-length \( \gamma \)-mENaC. In these cells, the nucleus was marked with DsRed. This was observed in 35% of the cells. In another population of mpkCCDc14 cells expressing eYFP-\( \gamma \)-mENaC (20% of the cells), as represented by the cell in the fluorescence micrographs shown in the middle row of Fig. 3, the bulk of eYFP emissions appeared in the nucleus to include the nucleoli. One possibility for these observations is that proteolytic processing of ENaC to release the NH2-terminal portion (tagged with eYFP) is more prevalent in this latter population. In a third population containing 45% of the mpkCCDc14 cells expressing eYFP-tagged \( \gamma \)-mENaC, emissions from the nucleus were not apparent, presumably reflecting cells having less ENaC processing (not shown).

Figure 4 documents the physiological consequences of the NH2-terminal portion of \( \gamma \)-mENaC targeting to the nucleus. Shown in Fig. 4A are representative macroscopic current traces before (denoted with arrow) and after amiloride (10 \( \mu M \)) for ENaC heterologously expressed in CHO cells in the absence and presence of additional coexpression of the cytosolic NH2- or COOH-terminal portions (as eGFP fusion proteins) of the \( \gamma \)-subunit. Coexpression of the NH2 but not COOH terminus suppressed ENaC activity. As summarized in Fig. 4B, the cytosolic NH2-terminal portion significantly decreased activity. (For these experiments, \( \gamma N \) was included at \( \sim 0.5 \times \) molar ratio compared with full-length subunits.)

In contrast to its effects on ENaC activity, targeting of the NH2-terminal portion of \( \gamma \)-mENaC to the nucleus, as shown in Fig. 5, did not decrease the activity of (or affect in any way) the voltage-gated Na\(^+\) channel Nav1.5. Shown in Fig. 5A are representative macroscopic current traces for Nav1.5 heterologously expressed in a CHO cell. Currents were evoked by step depolarization from a holding potential of \(-70\) to \(60\) mV in 10-mV increments. Figure 5B reports the macroscopic current-voltage relationship for Nav1.5 in the absence and presence of coexpression of the cytosolic NH2-terminal portion (as an eGFP fusion protein) of \( \gamma \)-ENaC. As summarized in Fig. 5C, coexpression of \( \gamma N \) had no effect on Nav1.5 activity. (As above, \( \gamma N \) was included in these experiments at \( \sim 0.5 \times \) molar ratio compared with full-length Nav1.5.)

As shown in Fig. 6A, the effects of coexpressing the NH2-terminal, cytosolic portion of \( \gamma \)-mENaC on ENaC activity is dose dependent with molar ratios (compared with full-length channel subunits) \( \geq 0.5 \times \), significantly decreasing ENaC activity. In contrast, as shown in Fig. 6B, coexpression of the COOH-terminal portion of \( \gamma \)-ENaC at similar molar ratios has no effect on activity.
As shown in Fig. 7, quantifying effects on the functional half-life of membrane ENaC subunits in the presence of an acute translation block to the synthesis of new channel protein. The functional half-life of ENaC activity as the channel is degraded over time in the absence of ENaC at the plasma membrane in the absence of the synthesis of new channel protein. This is in good agreement with the findings of others (43, 48). Coexpression of γ-mENaC as eGFP fusion proteins. Current was elicited with a voltage ramp from 60 to −100 mV from a holding potential of 40 mV. (CHO cells not transfected with ENaC subunits have little background leak current and no amiloride-sensitive Na+ current (43, 48).) Because accumulation of the NH2-terminal segment of γ-mENaC in the nucleus had functional consequences, we felt it important to detail the specific residues involved in targeting this portion of the channel to the nucleus. As noted above, both the extreme NH2 terminus of γ-subunits and the regions just preceding the first transmembrane domains in these subunits contain amino acid tracks with conserved basic residues that fit monopartite sequences in NLS (10, 14, 38). To test the functional importance of these conserved basic residues in trans-regulation of active ENaC at the membrane and targeting of the NH2 terminus of γ-mENaC to the nucleus, we sequentially charge-neutralized the conserved basic residues with alanine substitution. Specifically, we engineered seven different transcripts, as listed in Fig. 8, replacing K6 and K8 (transcript 1); K12 and K13 (transcript 2); R42 and R43 (transcript 3); R52 and R53 (transcript 4); K6, K8, K12, and K13 (transcript 5); R42, R43, R52, and R53 (transcript 6); and K6, K8, K12, K13, R43, R45, R52, and R53 (transcript 7). As shown by the representative fluorescence micrographs in Fig. 8, when these alanine-substituted transcripts were heterologously expressed in CHO cells, neutralization of the basic residues preceding the first transmembrane domain (R42, 43, 52, and 53; transcript 6) but not those in the extreme NH2 terminus (K6, 8, 12, and 13; transcript 5) disrupted targeting to the nucleus. Similarly, simultaneous substitution of the conserved basic residues in the extreme NH2 terminus and just preceding the first transmembrane completely disrupted targeting of the NH2-terminal portion of γ-mENaC to the nucleus.

These results are consistent with the cytosolic, NH2-terminal portion of γ-mENaC targeting to the nucleus and, consequently, affecting ENaC activity. We wondered about the mechanism associated with such regulation. The fusion protein containing the NH2 terminus of γ-mENaC, as well as the full-length mENaC subunits, was coexpressed as exogenous cDNA plasmids. Thus we thought decreased activity unlikely to result from direct regulation by γN of ENaC expression from exogenous transcripts driven by the CMV promotor but rather possibly result from trans-regulation by γN localized to the nucleus of some signaling molecule/pathway ultimately influencing posttranslational modulation of the channel activity. To begin identifying the final mechanism of regulation, we quantified the actions of coexpressing γN-eGFP with full-length subunits in the presence of an acute translation block to quantify effects on the functional half-life of membrane ENaC. As shown in Fig. 7, A and B, a sudden block of translation leads, as expected, to a time-dependent decrease in mENaC activity as the channel is degraded over time in the absence of the synthesis of new channel protein. The functional half-life of ENaC at the plasma membrane in the absence of γN was ~5 h. This is in good agreement with the findings of others reporting the functional half-life of membrane ENaC (8, 39, 51). Coexpression of γN-eGFP with full-length channel subunits markedly decreased the half-life to 2 h, demonstrating that the functional channel at the plasma membrane turns over more quickly in the presence of the NH2 terminus of γ-mENaC targeting to the nucleus.

Because accumulation of the NH2-terminal segment of γ-mENaC in the nucleus had functional consequences, we felt it important to detail the specific residues involved in targeting this portion of the channel to the nucleus. As noted above, both the extreme NH2 terminus of γ-subunits and the regions just preceding the first transmembrane domains in these subunits contain amino acid tracks with conserved basic residues that fit monopartite sequences in NLS (10, 14, 38). To test the functional importance of these conserved basic residues in trans-regulation of active ENaC at the membrane and targeting of the NH2 terminus of γ-mENaC to the nucleus, we sequentially charge-neutralized the conserved basic residues with alanine substitution.
Figure 9A summarizes the effects of a mutating monopartite NLS on targeting γN to the nucleus. While replacing the basic residues just preceding the first transmembrane (transcript 6) significantly decreased targeting to the nucleus, a large percentage (~70%) of this mutant remained correctly targeted to the nucleus similar to γN not substituted. In contrast, removing all basic residues in the presumptive monopartite NLS throughout γN (transcript 7) completely disrupted targeting to the nucleus, resulting in levels similar to background. This suggests that the region just preceding the first transmembrane contains the bulk of the NLS targeting γN to the nucleus but that additional information from sequences at the extreme NH2 terminus also contributes to this targeting. As expected then, substitution of the basic residues just preceding the first transmembrane domain (transcript 6), as well as neutralization of all basic residues in the putative monopartite NLS in γN (transcript 7), abrogated, as shown in Fig. 9B, the effects of γN on ENaC activity. In contrast, replacing only the charged residues in the putative NLS in the extreme NH2 terminus (transcript 5) has no effect on the negative actions of γN on ENaC activity.

DISCUSSION

The current results demonstrate that the cytosolic, NH2 terminus of γ-mENaC when removed from the core of the channel complex and expressed as a fusion protein targets to the nucleus. We also show that, in some instances, the NH2 terminus from full-length γ-ENaC is capable of similar targeting to the nucleus in native epithelial cells. Such targeting is similar to the targeting to the nucleus of the cytosolic portions of Notch receptors and the COOH-terminal tail of PC-1 following the ordered cleavage and release of these polypeptides from the core protein during signal transduction (5, 13, 18, 20, 36). Also, similar to signaling via Notch receptors and PC-1 involving cleavage, targeting of the NH2 terminus of γ-mENaC to the nucleus influences cellular activity. Specifically, accumulation of the NH2 terminus of γ-mENaC in the nucleus decreases activity of ENaC within the plasma membrane in a dose-dependent manner by decreasing the half-life of the functional channel. Modulation of active ENaC within the plasma membrane, the target, by γN localized to the nucleus is in trans, suggesting that release of the NH2 terminus of
γ-mENaC from the channel core initiates feedback regulation stemming from the nucleus. Regulation by nuclear-localized γN may be specific to ENaC for, to the limit that it was tested here, we found no effect of γN on a different Na⁺ channel, Nav1.5; however, specificity largely remains an open question. The NH₂ terminus of γ-mENaC localizes to the nucleus when a latent NLS in the cytosolic region of the protein just preceding the first transmembrane domain becomes the dominant targeting signal, as may happen when this portion of the channel is cleaved and released from the channel core.

These observations are consistent with the possibility that ENaC may serve dual roles within a cell. Clearly, this protein complex has an important ion channel function critical to vectorial Na⁺ transport across polarized epithelium (7, 22, 30, 32, 35). The current results, however, suggest that ENaC also may serve an important, but undera-

Fig. 8. Conserved basic residues in the region just preceding the first transmembrane domain in γ-mENaC contain the bulk of the NLS. Fluorescence micrographs (and corresponding overlays on images collected with white light) of CHO cells expressing γN as an eGFP-fusion protein with and without alanine substitutions of conserved basic amino acids in the extreme NH₂-terminus and just preceding the first transmembrane domain are shown. Substituted residues in the cytosolic NH₂-terminal portion of γ-mENaC are denoted in the primary sequence shown at the top: 7 different transcripts (1–7) were assayed and compared with unsubstituted γN.

Fig. 9. Disruption of the NLS in the cytosolic NH₂-terminal portion of γ-mENaC abolishes negative actions on ENaC activity. A: summary graph reporting the percentage (compared with total levels) targeting to the nucleus for γN and the 7 transcripts of γN containing substituted basic residues tested in Fig. 5. Control is background fluorescence in the nucleus for eGFP-tagged ENaC subunits in the absence of γN-eGFP. *Significantly less compared with unsubstituted γN; n = 10 for each measurement. B: summary graph of the mean amiloride-sensitive, macroscopic ENaC current density from CHO cells expressing mENaC alone and with γN or mutants (5, 6, and 7) of γN containing substituted basic residues. All other conditions are identical to those in Fig. 2A. *Significantly less compared with ENaC alone; n = 9 for each measurement.
ppreciated, role in signaling. More precisely, the current results seem to indicate that ordered cleavage of ENaC within the plasma membrane is poised to initiate signal transduction that can influence nuclear events. Intuitively, such a possibility is attractive for ENaC and related ion channel proteins in the ENaC/Deg superfamily are distinct compared with other ion channel proteins. Specifically, most of the ENaC protein is extracellular with the quaternary structure of these protein complexes being reminiscent more of membrane receptors than other ion channels.

We remain guarded yet of fully accepting the idea that membrane ENaC serves a signaling role in addition to functioning as an ion channel, for several key questions remain unanswered. Preeminent among these is whether ENaC cleavage does indeed result in the release and dissociation of the NH2 terminus of the γ-subunit in a manner that allows it to move independently of the remaining channel core. Most contemporary thinking is that pieces cleaved from ENaC remain attached/link to the channel core (reviewed in Ref. 33). However, this has not been studied in detail. Moreover, the current findings are consistent with some portion of full-length γ-ENaC targeting, possibly as a result of cleavage, to the nucleus in native epithelial cells. There is additional evidence emerging to support such a possibility, for a recent study finds that cleavage of α-ENaC results in dissociation of the NH2 terminus of this subunit, including the first transmembrane domain, from the channel core with the cleaved polypeptide capable of independent movement (26). While we report here the ramifications on ENaC activity of accumulating the NH2 terminus of γ-ENaC in the nucleus, details about how this portion of the channel, when in the nucleus, influences cellular activity to control the functional half-life of channels within the membrane remain obscure. Thus the current observations serve as important first steps in testing the possibility that cleavage of ENaC initiates a signaling cascade that has many parallels with Notch signaling; however, further study is needed before full acceptance of this idea.

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DISCLOSURES

No conflicts of interest are declared by the author.

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