Regulation of Epithelial Na\(^+\) Channel Activity by Conserved Serine/Threonine Switches within Sorting Signals*

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The PY and YXX\(\Phi\) motifs are canonical sorting signals involved in trafficking. Nedd4-2 and the \(\mu_2\)-subunit of the AP-2 complex target these motifs to facilitate internalization. Epithelial Na\(^+\) channel (ENaC) subunits contain both motifs in their cytosolic COOH termini where they overlap (S/T)PPXXY(S/T)\(\Phi\). Just preceding the PY and embedded within the YXX\(\Phi\) motifs are conserved serine/threonine. We test here whether these conserved Ser/Thr modulate ENaC activity by influencing the function of the internalization domains. We find that co-expression of dominant-negative dynamin (K44A) with ENaC increases channel activity. Conversely, co-expression of Nedd4-2 and epsin with ENaC decrease activity. Alanine substitution of the conserved Thr\(^{628}\) preceding the PY motif in \(\gamma\)-mENaC had no effect on basal activity. Channels with this mutation, however, responded to K44A and epsin but not Nedd4-2. Similarly, mutation of the proline repeat in the PY motif of \(\gamma\)-mENaC disrupted only Nedd4-2 regulation having no effect on regulation by K44A and epsin. Alanine substitution of the conserved Thr within the YXX\(\Phi\) motif of \(\gamma\)-mENaC (T635A) increased basal activity. Channels containing this mutation responded to Nedd4-2 but not K44A and epsin. Channels containing the T635(D/E) substitution in \(\gamma\)-mENaC did not have increased basal activity and responded to Nedd4-2 but not K44A. The double mutant T628A, T635A did not respond to Nedd4-2 or K44A. Mutation of Thr\(^{628}\) and Thr\(^{635}\) also disrupted ENaC precipitation with the \(\mu_2\)-subunit of the AP-2 complex. Moreover, the YXX\(\Phi\) motif, independent of the PY motif, was sufficient to target degradation with T635A disrupting this effect. These results demonstrate that the overlapping PY and YXX\(\Phi\) motifs in ENaC are, in some instances, capable of independent function and that the Ser/Thr just preceding and within these domains impact this function.

The epithelial Na\(^+\) channel (ENaC)\(^2\) is localized to the luminal plasma membrane of epithelial cells, particularly those involved in fluid absorption and epithelia surface hydration (1–3). As a consequence of this location and its function, ENaC activity is often limiting for Na\(^+\) absorption with corresponding effects on fluid movement. The critical role played by ENaC in terrestrial vertebrates has become apparent from the location mutations in this ion channel and its upstream regulators. Loss of function mutations result in inappropriate renal salt wasting in humans associated with electrolyte imbalance and decreased blood pressure (4–6). Moreover, in mice and possibly humans, loss of ENaC function also impairs fluid clearance of the fetal lung (7, 8). Gain of function mutations in ENaC and its upstream regulators, in contrast, result in inappropriate renal salt conservation associated with hypertension (2, 9). Recent evidence, moreover, suggests that gain of ENaC function may also lead to dry air spaces similar to that in cystic fibrosis (10).

ENaC is a heteromeric channel comprised of three similar but distinct subunits: \(\alpha\), \(\beta\), and \(\gamma\) (11, 12). ENaC is a member of the ENaC/Deg ion channel superfamily. ENaC subunits, similar to all members of the ENaC/Deg superfamily, have a common tertiary structure: two transmembrane domains separating a large extracellular loop from cytosolic NH\(_2\) and COOH termini.

ENaC activity reflects, in part, the membrane levels of this ion channel. All three ENaC subunits, \(\alpha\), \(\beta\), and \(\gamma\), contain a conserved PY motif (XPPXYY) in their COOH termini (13–16). This motif interacts with WW-domains, particularly, those in ubiquitin ligases, such as Nedd4 and Nedd4-2. Ubiquitinylation mediated by ubiquitin ligases targets proteins for internalization and/or degradation. The interaction of Nedd4 ubiquitin ligases with ENaC leads to ubiquitinylation of the channel with corresponding decreases in channel membrane levels and activity (15, 16). Disruption of the WW-interacting PY motif in \(\beta\)- and \(\gamma\)-hENaC are gain of function mutations that result in inheritable hypertension in humans (13).

Overlapping the PY motif in each of the three ENaC subunits is a putative tyrosine-based (YXX\(\Phi\)) where \(\Phi\) represents a residue with a hydrophobic side chain) endocytic/trafficking signal. The \(\mu_2\)-subunit of the plasma membrane clathrin-associated adapter complex AP-2, and the analogous \(\mu_3\)-subunit of the AP-1 complex associated with clathrin in the trans-Golgi network and endosome interact with proteins via YXX\(\Phi\) motifs (17–20). Shimkets and colleagues (21) have provided the only evidence, as yet, that YXX\(\Phi\) motifs in ENaC may be functionally relevant. These investigators demonstrated that co-expression of dominant-negative dynamin with ENaC increases channel activity. Dynamin is a GTPase that promotes budding of clathrin-coated vesicles.

Whereas PY and YXX\(\Phi\) motifs are common tyrosine-based sorting signals found in the cytosolic domains of many integral membrane proteins, they are infrequently found in the same protein (22, 23). When a protein does contain both types of sorting signals, they are most often separated in the linear amino acid sequence. However, several channel subunits, including ENaC subunits, \(\alpha\)-subunits of voltage-gated Na\(^+\) channels (Na\(_v\)), \(\beta\)-subunits (bartin) of voltage-gated CI\(^-\) channels, connexin 43, and some ether-a-go-go-related gene K\(^+\) channel subunits, contain a conserved domain, XPPXYYXX\(\Phi\), encompassing overlapping PY and YXX\(\Phi\) motifs (14, 16, 24–28). It currently is unclear if such overlapping sorting motifs function together as a single unit or whether each distinct motif has discrete and thus, separable function.

Immediately preceding the PY motif and imbedded within the YXX\(\Phi\)
motif of ENaC subunits are conserved serine/threonine residues (S/T)XTPRXYXS(S/T)φ. These residues can be differentially phosphorylated to influence ENaC activity with the prior but not latter Ser/Thr influencing Nedd4 binding (28–30).

We test here whether these conserved Ser/Thr differentially modulate ENaC activity and responses to proteins involved in trafficking. The current results are most consistent with the overlapping motifs in ENaC being capable of acting, in some instances, in a modular manner to regulate channel activity with the conserved Ser/Thr preceding and embedded with the PY and YXXφ motifs, respectively, differentially impacting discrete internalization/degradation mechanisms, or discrete steps within a single mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade and purchased from Sigma, BioMol, Fischer Scientific, and Tocris, unless noted otherwise. The mouse monoclonal anti-SOS and anti-AQP50 (μ3) antibodies used in Western blot analysis were from BD Transduction Laboratories. The Saccharomyces cerevisiae Western blot analysis were from BD Transduction Laboratories. The Saccharomyces cerevisiae (Mayo Clinic College of Medicine), D. Pearce (University of California, and jointly in

**Electrophysiology**—Whole cell macroscopic current recordings of recombinant mENaC expressed in CHO cells were made under voltage-clamp conditions using standard methods (31–34). Current through ENaC was the inward, amiloride-sensitive Na current with an extracellular bath solution of (in mM) 160 NaCl, 1 CaCl2, 2 MgCl2, and 10 HEPES (pH 7.4), and an intracellular pipette solution of (in mM) 120 CsCl, 5 NaCl, 2 MgCl2, 5 EGTA, 10 HEPES (pH 7.4), 2.0 ATP, and 0.1 GTP. 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 suite of software (Axon Instruments). Both a family of test pulses (500 ms each) stepping by 20-mV increments form a holding potential of 40 to 100 mV to −120 mV and voltage ramps (500 ms) from 60 to −100 mV were used to measure ENaC activity.

**Statistics**—Complementation frequency was compared using a z-test on proportions. All patch clamp data are presented as mean ± S.E. Unpaired data were compared using the appropriate t tests. p < 0.05 was considered significant.
FIGURE 1. Conserved Ser/Thr immediately before and within PY and YXX\(\phi\) motifs of ENaC differentially control channel activity. A, alignment of the conserved overlapping PY and YXX\(\phi\) motifs in the COOH termini of epithelial (ENaC) and voltage-gated (Na\(_\text{v}\)) Na\(^+\) channels, voltage-gated ERG K\(^+\) channels (K\(_{11.1}\) and 11.3), connexin 43, and barttin. Residue numbering is for \(\gamma\)-mENaC. [\(\beta\)-mENaC], \(\alpha\)-mENaC, \(\delta\)-mENaC, \(\alpha\_2\)-ENaC (top) or had \(\gamma\)-subunits with the T628A (and) and T635A (bottom) mutations. C, summary graph of ENaC activity reported as the amiloride-sensitive current density at \(-80\) mV for cells expressing wild-type mENaC and channels having the T628A and T635A mutations. Asterisk, versus mENaC. D, summary graph of ENaC activity reported as the amiloride-sensitive current density at \(-80\) mV for cells expressing wild-type mENaC and channels having the T613A and T620A mutations in their \(\beta\)-subunits. Asterisk, versus mENaC.

\(\alpha\)- and \(\beta\)-subunits with \(\gamma\)-subunits containing the T628A and the T635A substitutions, respectively. As shown in the summary graph in Fig. 1C, which shows the effect of dominant-negative dynamin on ENaC activity, whereas the \(\gamma\)-ENaC, increased activity, whereas, the Ser/Thr embedded within the YXX\(\phi\) motif in \(\gamma\)- but not \(\beta\)-ENaC increased activity.

**FIGURE 2. Regulation of ENaC by proteins involved in trafficking.** A, macroscopic current traces in voltage-clamped CHO cells before (left) and after (right) amiloride. Currents were elicited with 20 mV voltage steps from a holding potential of 40 to 100 mV down to \(-120\) mV. Cells were not transfected (top) or transfected with hENaC alone (top middle) and plus dynamin (bottom middle) and dominant negative (K44A) dynamin (bottom). B, summary graph of the effects of dynamin and K44A on ENaC at \(-80\) mV. Activity is reported as the amiloride-sensitive current density at this voltage. Asterisk, versus hENaC. C, summary graph of the effects of co-expression of dominant-negative dynamin (K44A), Ned4-2 (N4-2), and epsin with mENaC on channel activity at \(-80\) mV. Asterisk, versus mENaC.
Modular Retrieval Motifs Regulate ENaC

TABLE ONE

Regulation of wild-type and mutant ENaC by Nedd4-2, dominant-negative dynamin, and epsin

Activities are amiloride-sensitive current densities at −80 mV in pA/pF. The number of observations for each group are in parentheses.

|          | Control       | +Nedd4-2      | +K44A        | +Epsin        |
|----------|---------------|---------------|--------------|---------------|
| mENaC    | 196 ± 22 (16) | 27 ± (6)*     | 630 ± 142 (6)* | 118 ± 21 (17)* |
| T628A    | 165 ± 37 (8)  | 149 ± 52 (9)* | 490 ± 83 (5)* | 80 ± 17 (15)*  |
| T635A    | 470 ± 64 (14)*| 38 ± 13 (10)* | 440 ± 58 (15) | 460 ± 80 (12)* |
| T635D    | 132 ± 30 (9)  | 10 ± 6 (9)*   | 123 ± 16 (9)* | NT*           |
| 3P       | 228 ± 23 (9)  | 90 ± 21 (14)* | 600 ± 84 (7)* | 118 ± 29 (6)*  |

*a p < 0.05 versus group control.
*b p < 0.05 versus mENaC under similar conditions.
*NT, not tested.

FIGURE 3. Thr628 and Thr635 differentially affect ENaC regulation by trafficking proteins. Summary graph of the effects of K44A (black bars), epsin (white bars), and Nedd4-2 (dark gray bars) on activity of wild-type ENaC and ENaC having the T628A and T635A mutations in their γ-subunit. Control levels for each group are shown with light gray bars. Asterisk, versus control.

significantly increased hENaC activity. As shown in Fig. 2C (see also TABLE ONE), mENaC had a similar response with dominant-negative dynamin significantly increasing activity. Also shown in Fig. 2C are the effects of co-expression of Nedd4-2 (N4-2) and epsin on mENaC activity. Co-expression of Nedd4-2, as expected, significantly decreased ENaC activity. Co-expression of epsin with ENaC, similar to Nedd4-2 and in contrast to dominant-negative dynamin, significantly decreased channel activity. These results demonstrate that the activity of wild-type ENaC heterologously expressed in CHO cells is increased and decreased when proteins involved in endocytosis and/or transit to the late endosome, lysosome, and proteosome are disrupted and overexpressed, respectively.

Mutation of Thr628 and Thr635 Differentially Impact Regulation by Dynamin, Nedd4-2, and Epsin—To address the question of whether the conserved Ser/Thr preceding and within the PY and YXXφ motifs differentially impact regulation of ENaC by proteins involved in trafficking, we assayed the effects of dominant-negative dynamin (black bars), epsin (white bars), and Nedd4-2 (dark gray bars) on activity of wild-type ENaC and ENaC having the T628A and T635A mutations in their γ-subunit. Control levels for each group shown (see also TABLE ONE). TABLE ONE summarizes results from these experiments. Similar to wild-type channels, dominant-negative dynamin significantly increased activity of channels containing γT628A—3-fold. In contrast, dominant-negative dynamin had no effect on the activity of channels containing γT635A. Recall that steady-state activity of channels containing this latter substitution are already ~3-fold greater compared with activity of wild-type channels. In contrast to dominant-negative dynamin, Nedd4-2 affected channels having T635A in a manner identical to its effects on wild-type channels, significantly decreasing current, but had no effect on channels containing the T628A substitution. Co-expression of epsin significantly decreased activity of channels with T628A but not T635A. The response of T628A but not T635A channels to epsin, then, is similar to the response of wild-type channels. Thus, dominant-negative dynamin and epsin both affect wild-type and T628A channels but in opposite directions, and do not affect T635A channels. In contrast, Nedd4-2 affects wild-type and T635A channels but not T628A channels. Channels having the double mutation T628A,T635A, as expected, were insensitive to Nedd4-2 and dominant-negative dynamin (not shown; epsin not tested). These results demonstrate that the Ser/Thr preceding and within the PY and YXXφ motifs of γ-ENaC differentially modulate regulation of the channel by proteins involved in trafficking.

The effects of dominant-negative dynamin, Nedd4-2, and epsin on channels containing γ-subunits with alanine substitution of the three prolines (629–631) within the PY motif of γ-ENaC were insensitive to Nedd4-2 and dominant-negative dynamin (not shown; epsin not tested). These results demonstrate that the Ser/Thr preceding and within the PY and YXXφ motifs of γ-ENaC differentially modulate regulation of the channel by proteins involved in trafficking.
comparison, Thr_{635} does not appear to affect interactions with Nedd4-2 but rather likely impacts processes involving dynamin and epsin. While not yet definitive, this interpretation is consistent with the PY and YXX\phi motifs having separable function in some instances.

**YXX\phi Is Sufficient to Mediate \(\gamma\)-ENaC Retrieval and Degradation—** We next performed experiments that more directly tested the exciting possibility that the overlapping PY and YXX\phi motifs act, in some instances, independent of each other. Previously with a yeast one-hybrid complementation screen, we demonstrated that the COOH terminus of \(\gamma\)-ENaC localizes to the plasma membrane (35, 36). We also showed that deletion of the COOH terminus following the overlapping PY and YXX\phi motifs in \(\gamma\)-mENaC led to strong activation of these motifs resulting in retrieval and subsequent degradation. Building on these earlier observations, we asked here whether the YXX\phi motif within the COOH terminus of \(\gamma\)-mENaC alone was sufficient to target degradation. We also tested whether the conserved threonines preceding and within the PY and YXX\phi motifs, respectively, influenced degradation mediated by the overlapping motifs, and by the YXX\phi motif alone.

Fig. 5A shows a typical complementation screen where truncated SOS-\(\gamma\)ENaC_{637X} does not complement (top rows). This is in contrast to the fusion protein containing the entire COOH terminus of \(\gamma\)-ENaC (SOS-\(\gamma\)A568-end\(\alpha\)mENaC; see Refs. 35 and 36 and Fig. 5B), which complements. Similar to the truncation mutant, SOS-\(\gamma\)ENaC_{637X} expression of the deletion mutant \(\gamma\)ENaC_{A561-R632} removes the residues, including the PY motif, just preceding the YXX\phi motif, failed to complement. From earlier work (35, 36) we knew that SOS-fusion proteins containing \(\gamma\)ENaC_{637X} and \(\gamma\)ENaC_{A561-R632} express well but are targeted for degradation. We wondered whether the conserved threonines preceding (Thr_{628}) and within (Thr_{635}) the PY and YXX\phi motifs, respectively, influenced this signal-mediated degradation. As shown in Fig. 5A, and summarized in Fig. 5B, alanine substitution of Thr_{628} reversed the absence of complementation by the SOS-\(\gamma\)ENaC_{637X} fusion protein. Similarly, the SOS-\(\gamma\)ENaC_{A561-R632} fusion protein containing the T635A mutation complemented. As shown in the typical Western blot of Fig. 5C, these threonine mutations disrupted degradation of the fusion proteins. These results demonstrate that the YXX\phi motif in \(\gamma\)-mENaC, in certain circumstances, is sufficient to target degradation and that the conserved threonine within this motif modulates this activity.

**ENaC Interacts with the \(\mu_2\)-Subunit of the AP-2 Complex—** The AP-2 complex is an adapter that couples proteins to clathrin. The \(\mu_2\)-subunit of AP-2 directly interacts with proteins at the YXX\phi motifs (17, 18). Epsin, which binds both clathrin and AP-2, also interacts with ubiquitylated proteins via an ubiquitin-interacting motif to couple these latter proteins to clathrin/AP-2 (45, 46). Thus, there are several ways that ENaC may possibly associate with AP-2. The representative Western blots (n = 3) in Fig. 6 tested whether ENaC interacts with the \(\mu_2\)-subunit of the AP-2 complex, and whether the conserved Ser/Thr preceding and within the PY and YXX\phi motifs influence this interaction. The top blot in this Fig. 6 contains whole cell lysate from untransfected cells (con) and cells expressing His- and Myc-tagged \(\alpha\)-ENaC plus Myc-tagged \(\beta\) and Myc-tagged wild-type and T628A, T635A mutant \(\gamma\)-ENaC. The blot was probed with anti-Myc antibody. The lower blot contains His-tagged complexes precipitated with ProBond nickel chelating resin from the respective whole cell lysates. The lower blot was probed with anti-\(\mu_2\) antibody and demonstrates that the \(\mu_2\)-subunit of the AP-2 complex interacts with ENaC. Substitution of Thr_{628} and Thr_{635}...
resulted in a qualitative decrease in the amount of μ2-subunit interacting with ENaC. These findings are consistent with the ideas that ENaC interacts with the AP-2 complex and that conserved Ser/Thr within the channel impact this interaction.

Substitutions of Thr628 and Thr635 in γ-ENaC are phosphorylated by cytosolic kinases with MAPK 1/2 targeting the former residue to facilitate Nedd4 binding and subsequent down-regulation of channel activity. In contrast, phosphorylation of Thr635, by an as yet defined kinase, appears not to affect Nedd4 binding, although it does impact channel activity (26, 28). We substituted Thr635 with Glu and Asp to test the idea that residues that potentially mimic phosphorylation at this site impact ENaC activity and differentially affect regulation by Nedd4-2 and dominant-negative dynamin. Fig. 7 summarizes activity of ENaC containing γγγγ and γγγγγγγγ in the absence and presence of dynamin, K44A, and Nedd4-2. Unexpectedly, neither the T635E nor the T635D substitution affected basal activity. However, the T635D (T635E not tested) substitution did disrupt regulation by dominant-negative dynamin but not Nedd4-2 with the latter still significantly decreasing activity.

**DISCUSSION**

Several novel observations are made in the current studies. Epsin decreased ENaC activity. The YXXϕ motif in γ-ENaC was proven sufficient, even in the absence of the PY motif, to mediate ENaC degradation in some cases. Mutation of conserved Ser/Thr residues just before and within the PY and YXXϕ motifs of β- and γ-ENaC differentially impacted the steady-state current, and responses to proteins, including dominant-negative dynamin, epsin, and Nedd4-2, involved in trafficking. What we believe most important about the current findings, however, is the insight they provide with regards to regulation of ENaC by sorting signals. Our results suggest that in the CHO cell model, the two distinct overlapping tyrosine-based sorting signals, the PY and YXXϕ motifs, found in the COOH terminus of ENaC subunits, as well as other types of channels, have separable function with conserved Ser/Thr influencing this function. It is possible that these sorting motifs act in concert at different steps along a common sorting route or are involved in separate mechanisms for sorting the channel.

The observations that alanine substitutions for Thr628 and Thr635 in γ-ENaC and Thr613 and Thr620 in β-ENaC have distinct effects on steady-state activity are in a general sense consistent with the findings of others (29, 30, 47). In addition, they agree with the idea that these residues are phosphorylation targets with their phosphorylation state playing an important role in modulating channel activity via regulating how ENaC is perceived by proteins involved in trafficking. In 2001, Chigaev and colleagues (29) made the initial observation that the threonines within the YXXϕ motifs of β- and γ-ENaC can be phosphorylated. In contrast to the present study, alanine substitution of these sites did not affect basal rENaC activity when the channel was heterologously expressed in Xenopus laevis oocytes. Substitution, however, did limit the maximal channel activity achieved by a combination of co-expression of serum and glucocorticoid-inducible kinase (Sgk) and low Na +. Schild and colleagues (47) made a similar observation that alanine substitution of the threonine within the YXXϕ motif of γ-ENaC had little effect on steady-state rENaC currents when the channel was heterologously expressed in X. laevis oocytes. We speculate that these differences may reflect differences in the regulation of basal ENaC activity in CHO cells and X. laevis oocytes. This is not to say that the fundamental mechanisms involved in ENaC trafficking and regulation of activity are different in these two models of study but rather that the dynamic equilibrium between the component proteins involved in ENaC trafficking or the steady-state phosphorylation of the channel at conserved Ser/Thr within sorting motifs may differ in oocytes and CHO cells. It is unclear which model system better offers a true reflection of the prevalent pathways for retrieval and degradation in native epithelial cells. Thus, translation of the current findings to regulation of ENaC in native epithelia remains to be fully explored.

Similar to earlier studies (30, 47), we find that substitution of the threonine immediately preceding the PY motif (T613A) but not that within the YXXϕ motif (T620A) of β-ENaC increased basal activity. Thus with respect to threonine mutations in β-ENaC, CHO cells and oocytes yield similar results. The former residue in β-ENaC (Thr613 in mENaC) and the analogous residue in γ-ENaC (Thr628 in mENaC) are phosphorylated by MAPK 1/2 (30). Phosphorylation increases Nedd4 binding to ENaC. The current results demonstrating that channels having the T628A mutation no longer respond to regulation by Nedd4-2 are consistent with this idea. Interestingly, these channels, whereas insensitive to regulation by Nedd4-2, did not have greater basal activity. This suggests that in CHO cells endogenous Nedd4 ubiquitin ligases may have low activity at rest. An alternative speculation is that the resting phosphorylation state of Thr628 in γ-ENaC in oocytes and CHO cells differs. Indirect evidence better supports the latter speculation for co-expression of Sgk with ENaC in CHO cells increases activity. A well documented mechanism by which Sgk increases ENaC activity is to disrupt Nedd4-2 down-regulation of the channel (41, 43). Nevertheless, the important point brought out by the current results is that mutation of Thr628 and Thr635 differentially affect basal activity. We interpret this to mean that these threonines or rather possibly their phosphorylation state act as switches controlling ENaC activity perhaps by influencing how channel signaling motifs are perceived by proteins involved in trafficking.

The observations that channels containing T628A and T635A respond differently to Nedd4-2, epsin, and dynamin are consistent with this interpretation. Because channels containing the T628A mutation behaved, for the most part, similar to those having the 3P mutation in the γ-subunit with respect to regulation by Nedd4-2, epsin, and dominant-negative dynamin, we propose that Thr628 influences Nedd4-2 interactions with ENaC. This is the same conclusion drawn by Shi and colleagues (30) in their earlier study. However, we increase understanding by demonstrating here that Thr635, whereas possibly being important to Nedd4-2 interactions with ENaC, plays less of a dominant role in trafficking involving epsin and dynamin. It is unclear at this time if Nedd4-2, epsin, and dynamin have roles at different sites along a common trafficking pathway or are components of distinct trafficking pathways with respect to ENaC. Regardless of whether these trafficking proteins are involved in one or more pathways with respect to regulation of ENaC, we can state that in CHO cells, Thr628 and Thr635 play important roles in the actions of Nedd4-2, and dynamin and epsin, respectively. As noted above, how well these findings in CHO cells translate to the situation in native epithelia remains to be determined.

Our observations raise two possibilities. First, that Thr628 and Thr635 influence ENaC interactions with trafficking proteins via effects at different binding sites within the channel. For instance, Thr628 could influence Nedd4-2 binding to the PY motif and Thr635 could influence binding of the μ2-subunit to the YXXϕ motif. The finding reported here that this latter motif is functional independent of the PY motif, in certain settings, lends support to this idea. Moreover, that ENaC can interact

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3 J. D. Stockand and L. LaGrange, unpublished observation.
with the μ2-subunit of the AP-2 complex is also consistent with this idea. An equally plausible alternative is that Thr628 influences Nedd4-2 binding to the channel with subsequent epsin interaction with ubiquitylated ENaC via the ubiquitin-interacting motif being influenced by Thr635. Importantly, with either mechanism, these two threonines act as molecular switches controlling ENaC sensitivity to trafficking proteins.

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