Nedd4-2 Induces Endocytosis and Degradation of Proteolytically Cleaved Epithelial Na\(^+\) Channels*

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As a pathway for Na\(^+\) reabsorption, the epithelial Na\(^+\) channel ENaC is critical for Na\(^+\) homeostasis and blood pressure control. Na\(^+\) transport is regulated by Nedd4-2, an E3 ubiquitin ligase that decreases ENaC expression at the cell surface. To investigate the underlying mechanisms, we proteolytically cleaved/activated ENaC at the cell surface and then quantitated the rate of disappearance of cleaved channels using electrophysiological and biochemical assays. We found that cleaved ENaC channels were rapidly removed from the cell surface. Deletion or mutation of the Nedd4-2 binding motifs in \(\alpha\), \(\beta\), and \(\gamma\)ENaC dramatically reduced endocytosis, whereas a mutation that disrupts a YXXO endocytosis motif had no effect. ENaC endocytosis was also decreased by silencing of Nedd4-2 and by expression of a dominant negative Nedd4-2 construct. Conversely, Nedd4-2 overexpression increased ENaC endocytosis in human embryonic kidney 293 cells but had no effect in Fischer rat thyroid epithelia. In addition to its effect on endocytosis, Nedd4-2 also increased the rate of degradation of the cell surface pool of cleaved \(\alpha\)ENaC. Together, the data indicate that Nedd4-2 reduces ENaC surface expression by altering its trafficking at two distinct sites in the endocytic pathway, inducing endocytosis of cleaved channels and targeting them for degradation.

The epithelial Na\(^+\) channel ENaC forms a pathway for Na\(^+\) reabsorption across epithelia, including the kidney, lung, and colon. Therefore, it plays a critical role in Na\(^+\) homeostasis and blood pressure control (reviewed in Refs. 1 and 2). Defects in ENaC function or regulation cause inherited forms of hypertension and hypotension (3) and may contribute to the pathogenesis of lung disease in cystic fibrosis (4).

ENaC is regulated by Nedd4-2, a HECT domain E3 ubiquitin ligase that decreases ENaC expression at the cell surface (5—7). This regulation requires the binding of Nedd4-2 WW domains to PY motifs (PPXXYXXL) located in the C terminus of each of the three subunits that form the channel (\(\alpha\), \(\beta\), and \(\gamma\)ENaC). Mutations in the PY motifs of \(\beta\) or \(\gamma\)ENaC disrupt binding, causing Liddle syndrome (8—10). In this inherited form of hypertension, increased expression of ENaC at the cell surface results in excessive renal Na\(^+\) reabsorption (7, 8, 11). Binding is also modulated by aldosterone and vasopressin via serum and glucocorticoid-regulated kinase and protein kinase A, respectively; both kinases phosphorylate Nedd4-2, which reduces its binding to ENaC (6, 12, 13).

However, the mechanism by which Nedd4-2 reduces ENaC surface expression is uncertain. It is possible that Nedd4-2 regulates ENaC trafficking in the biosynthetic pathway, targeting it for degradation in the proteasome. Consistent with this model, localization of Nedd4-2 at the cell surface is not required for Nedd4-2 to inhibit ENaC (14). Moreover, proteasome inhibitors decrease ENaC degradation (15—17) and increase ENaC surface expression. Alternatively, Nedd4-2 could regulate ENaC in the endocytic pathway, altering ENaC endocytosis and/or targeting to lysosomes for degradation. This model is suggested by recent data from our laboratory and others indicating that Nedd4-2 binds to ENaC at the cell surface, where it catalyzes ubiquitination of each ENaC subunit (18, 19).

In this work, we investigated the mechanism(s) by which Nedd4-2 reduces ENaC surface expression. To overcome technical hurdles that have hindered progress in this area, we developed a novel strategy; we took advantage of the observation that ENaC is activated by proteolytic cleavage at specific sites in the extracellular domains of the \(\alpha\) and \(\gamma\) subunits (20—23). Using electrophysiological and biochemical assays, we tested the effect of Nedd4-2 on endocytosis and degradation of proteolytically cleaved channels.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Human \(\alpha\), \(\beta\), \(\gamma\)ENaC (24, 25) and Nedd4-2 (12) in pMT3 were cloned as described previously. Mutations were generated by site-directed mutagenesis (QuickChange; Stratagene). \(\alpha\)-FLAG was generated by insertion of a FLAG epitope (DYKDDDDK) at the C terminus (7). Small interfering RNA (siRNA)\(^3\) against Nedd4-2 was obtained from Qiagen and characterized previously (26).

Electrophysiological Endocytosis Assay—Fischer rat thyroid (FRT) cells cultured on permeable filter supports were transfected (TFX50; Promega) (12) with the following human ENaC subunits: \(\alpha\) or \(\alpha_{1\text{Cl}}\) (R177A, R178A), \(\beta\), and \(\gamma\) or \(\gamma_{1\text{Cl}}\) (R135A, R137A, R138A, R178W, R180A, K181A). For overexpression experiments, the cells were cotransfected with 0.23 \(\mu\)g of each ENaC subunit along with either green fluorescent protein

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\(^{3}\) The abbreviations used are: siRNA, small interfering RNA; FRT, Fischer rat thyroid; GFP, green fluorescent protein; PBS, phosphate-buffered saline; HEK, human embryonic kidney.
(GFP, control) or Nedd4-2 (0.3 µg). For RNA interference experiments, cells were cotransfected with 0.27 µg of each ENaC subunit and siRNA against GFP (control) or Nedd4-2 (0.2 µg) (26). In some studies, ENaC subunits contained the following mutations: αV644A, βR566X, βY620A, βT616–618A, γV627A, or γT629A.

Following transfection, the cells were cultured for 48 h as described previously (12). Short circuit current was measured using modified Ussing chambers (Warner Instrument Corp.). The apical and basolateral surfaces were bathed in 135 mM NaCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 2.4 mM K2HPO4, 0.6 mM KH2PO4, 10 mM HEPES, pH 7.4, at 37 °C. Amiloride-sensitive short circuit current was determined as the current difference with and without amiloride (10 µM) in the apical bathing solution. To proteolytically cleave and activate ENaC at the cell surface, trypsin (10 µg/ml) was added to the apical membrane for 10 min and then removed by vigorous washes with ~12 volumes of the apical chamber. Amiloride-sensitive current was measured every 5 min by addition of amiloride (10 µM) to the apical membrane followed by wash out of amiloride.

Biochemical Endocytosis Assay—HEK 293T cells were transfected (Lipofectamine 2000; Invitrogen) (18) with αC1.2-FLAG (R175A, R177A, R178A, R181A, R190A, R192A, R201A, R204A, C-terminal FLAG epitope), β, and γENaC. ENaC was cotransfected with cDNA encoding Nedd4-2, dominant negative Nedd4-2 (corresponding to amino acids 1 and 60–479 with Ser-221, Thr-246, and Ser-327 phosphorylation sites mutated to Ala), or GFP; or with siRNA against Nedd4-2 or GFP (quantities indicated in the figure legends). In some experiments, ENaC subunits contained the following mutations: βR566X, βV620A, βP616–618A, or γV629A. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10 µM amiloride. 48 h after transfection, the cells were washed with phosphate-buffered saline (PBS)-CM (PBS with 1 mM MgCl2 and CaCl2) and then incubated with trypsin (5 µg/ml) for 5 min at 37 °C. The cells were washed three times with PBS-CM to remove trypsin, incubated at 37 °C for times between 0 and 60 min to allow endocytosis of cleaved channels, and then placed on ice. Cleaved channels remaining at the cell surface were labeled with biotin, as previously described (7). Briefly, the cells were incubated with 0.5 mg/ml Sulfo-NHS-biotin (Pierce) in PBS-CM for 30 min on ice and then quenched with 100 mM glycine in PBS-CM for 10 min on ice. The cells were lysed in Nonidet P-40 lysis buffer (0.4% sodium deoxycholate, 1% Nonidet P-40, 63 mM EDTA, 50 mM Tris-HCl, pH 8, and protease inhibitor mixture). Biotinylated (cell surface) proteins were isolated by incubating the cell lysate with immobilized NeutrAvidin beads (Pierce) for 12 h at 4 °C. Following separation by SDS-PAGE, biotinylated αENaC was detected by immunoblot with anti-FLAG M2 monoclonal antibody-peroxidase conjugate and quantitated by densitometry.

Degradation Assay—HEK 293T cells were transfected with α-FLAG, β, and γENaC with Nedd4-2 or GFP. 48 h later, cells were biotinylated as above and then incubated at 37 °C for times between 0 and 80 min. Following lysis of the cells in Nonidet P-40 lysis buffer, biotinylated αENaC was isolated with NeutrAvidin beads, separated by SDS-PAGE, and detected by immuno

![FIGURE 1. Trypsin cleaves and activates uncleaved ENaC on cell surface. A and B, representative short circuit current traces in FRT epithelia transfected with αENaC (wild-type or αC), βENaC, and γENaC (wild-type or γC) (0.33 µg each). Each channel was activated with a brief exposure to trypsin. Over time, cleaved/active channels were removed from the cell surface by endocytosis. The rate of disappearance of cleaved channels from the cell surface is quantitated as an assay of endocytosis.](Image)

RESULTS

Proteolytic Cleavage of Cell Surface ENaC—In Fig. 1A, we transfected FRT epithelia with α, β, and γENaC and measured the short circuit current blocked by amiloride as an assay of ENaC activity. ENaC activity requires proteolytic cleavage of the extracellular domains of the α and γ subunits. The basal amiloride-sensitive current (Fig. 1A) reflects ENaC channels cleaved in the biosynthetic pathway (27, 28). Addition of trypsin to the extracellular solution increased amiloride-sensitive current 2-fold, reflecting acute proteolytic cleavage/activation of a pool of uncleaved (inactive) channels at the cell surface (Fig. 1A). This finding is consistent with previous work (7, 23, 27). In Fig. 1B, we mutated a consensus site for cleavage by furin in αENaC (αC1.2, R177A/178A), and in γENaC we mutated sites (γC3) for furin (R135/137/138A) and CAP1 (R178W, K179A, R180A, K181A) (27, 29). By preventing cleavage, these mutations nearly abolished basal amiloride-sensitive current. However, trypsin activated the mutant channel, generating a large amiloride-sensitive current. This presumably occurred via trypsin cleavage at remaining Arg or Lys residues adjacent to the mutated furin and CAP1 sites.

Quantitation of Endocytosis of Proteolytically Cleaved ENaC—Expression of the αC1βγC3 mutant ENaC in FRT cells provided a strategy to measure the rate of ENaC endocytosis (Fig. 1C). A pool of channels at the cell surface is proteolytically cleaved and activated with a brief exposure to trypsin. Over time, cleaved/active channels are removed from the cell surface by endocytosis, resulting in decreased amiloride-sensitive current. The rate of decrease in current reflects the rate of ENaC endocytosis. During this time course, newly synthesized channels also undergo endocytosis, but they are uncleaved/inactive and therefore do not contribute significantly to the current. Fig. 2A shows a representative current trace. Trypsin activated a large amiloride-sensitive current (arrowheads indicate
 brief addition of amiloride to quantitate ENaC current). Following removal of trypsin, amiloride-sensitive current decreased over time. Average data are shown in Fig. 2C, which is a plot of amiloride-sensitive current (relative to current immediately after trypsin removal) versus time after trypsin removal (error bars are hidden by the symbols), and data at 15 min are shown in Table 1. Current decreased rapidly over the first 15 min, followed by a much slower decrease. This suggests that cleaved ENaC is rapidly removed from the cell surface. The slower phase could represent a second more stable population that cleaved ENaC is rapidly removed from the cell surface. We coexpressed cotransfected FRT cells with αC1 and γC1 along with a mutant β subunit. In Fig. 2, B and C, we tested βR566X, which deletes most of the C terminus, including the PY motif. Following activation by trypsin, there was a gradual decline in amiloride-sensitive current. However, the rate of decrease was dramatically slower than for the wild-type β subunit. This difference between the curves was mainly in the initial rapid phase, which was largely absent for βR566X (Fig. 2C). In contrast, the curves for wild-type and βR566X paralleled one another in the later phase. When trypsin was added to the bathing solution a second time, there was an increase in ENaC current, albeit much smaller than for wild-type βENaC (Fig. 2B). Thus, Liddle syndrome mutations decrease ENaC exocytosis, consistent with previous work (30–32).

In Fig. 2D, we tested a missense mutation within the βENaC PY motif (βY620A) that also disrupts binding to Nedd4-2. This mutation slowed the rate of decrease in trypsin-activated ENaC current, similar to βR566X. Equivalent mutations of the PY motifs in αENaC (Y644A) and γENaC (Y627A) also slowed the decline in current (Fig. 2, E and F) although, interestingly, mutation of the αENaC PY motif had a smaller effect than the mutations in β or γENaC. Together, the data suggest that Liddle syndrome mutations decrease the rate of ENaC endocytosis.

Biochemical Endocytosis Assay—As a second strategy to quantitate ENaC endocytosis, we used a biochemical assay to measure the rate of removal of proteolytically cleaved channels from the cell surface. We coexpressed α, β, and γENaC in HEK 293T cells; the α subunit contained a FLAG epitope at the C terminus. To detect αENaC at the cell surface, we biotinylated cell surface proteins, isolated them with NeutrAvidin beads, and then immunoblotted with anti-FLAG antibody. For wild-type αENaC, we detected a 90-kDa band corresponding to the full-length protein and a 65-kDa band corresponding to the C-terminal cleavage fragment (Fig. 3A), consistent with previous work (7, 33). To prevent cleavage by furin, we mutated the two furin consensus sites (αC1.2), which has a similar effect on ENaC current as mutation of a single furin site (αC1.1) (34). This eliminated the 65-kDa band (Fig. 3A). However, extracellular trypsin (5 μg/ml for 5 min) cleaved αC1.2, generating a 65-kDa band very similar in mass to the cleaved form of wild-type αENaC (Fig. 3A). After removing trypsin from the extracellular medium, we measured the rate of disappearance of the 65-kDa band from the cell surface as an assay of ENaC endocytosis (Fig. 3A).
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**Figure 3. Biochemical endocytosis assay.** HEK 293T cells were transfected with the following ENaC subunits: α-FLAG (wild-type or αC1-2), β (wild-type, R566X, or Y620A), and γ (5.33 μg each). 48 h after transfection, cells were treated with or without trypsin (5 μg/ml for 5 min), and then cell surface α-FLAG was biotinylated, isolated with NeutrAvidin beads, and detected by immunoblot (anti-FLAG). A and B, representative immunoblots. In B, following removal of trypsin from the medium, cells were incubated for 0–60 min at 37 °C prior to biotinylation. Data are quantitated (densitometry) in C; plot of cleaved α-FLAG at the cell surface versus time at 37 °C (relative to 0 min) (mean ± S.E., n = 3–8 for each data point).

1C shows model). Because newly synthesized channels undergoing exocytosis are 90 kDa, we were able to distinguish them from cleaved channels undergoing endocytosis.

In Fig. 3B, top panel, we generated a pool of trypsin-cleaved αC1-2-FLAG at the cell surface, incubated the cells at 37 °C for 0–60 min to allow endocytosis, and then biotinylated channels remaining at the cell surface. We observed a rapid decrease in the 65-kDa cleaved band at the cell surface (Fig. 3B, average data are quantitated in Fig. 3C); 50% was removed by 15 min and nearly all was removed at 60 min. Thus, the half-life of cleaved αENaC at the cell surface was short (15 min). In contrast to the cleaved band, the quantity of the full-length 90-kDa band at the cell surface reflects the net contributions of both endocytosis and exocytosis of full-length αENaC. The quantity of this band increased at 5 min (2.1 ± 0.1-fold, n = 3), likely resulting from exocytosis of newly synthesized channels. There was little change in the density of this band at later time points, indicating that a steady state between endocytosis and exocytosis was reestablished. As a control, there was no change in the quantity of αC1-2-FLAG in the total cellular lysate during the course of the experiment (Fig. 3B, bottom panel). Minimal cleaved αC1-2-FLAG was observed in the total lysate, indicating that only a small fraction of total ENaC in the cell was expressed at the cell surface, consistent with previous work (7).

In Fig. 3B, right panels, and 3C, we tested the effect of Liddle syndrome mutations on endocytosis. Deletion of the C terminus of βENaC (R566X) decreased the rate of disappearance of the cleaved 65-kDa band from the cell surface. At 15 min, there was no significant decrease in surface expression and the half-life lengthened to >60 min. Mutation of the PY motif (β₃Y₆20A) had a similar effect (Fig. 3C). Together with the results of the electrophysiological assay, we conclude that proteolytically cleaved/active ENaC is rapidly removed from the cell surface and that Liddle syndrome mutations dramatically slow the rate of removal.

**Nedd4-2 Binding Motif Mediates ENaC Endocytosis**—The PPXYXXL sequence mutated in Liddle syndrome fits the consensus for two motifs that have the potential to mediate ENaC endocytosis. First, it fits the YXXØ (Ø indicates hydrophobic amino acids) motif for endocytosis in clathrin-coated pits. This motif mediates endocytosis of the transferrin receptor (35). Second, it fits the PPXY PY motif consensus that binds to proteins containing WW domains, including Nedd4-2 (36). The Tyr (β₃Y₆20A) is common to both motifs; mutation of this residue decreased ENaC endocytosis (Figs. 2D and 3C). Previous work indicates that the Leu contributes to WW domain binding and is also part of both motifs (37, 38). To distinguish the relative contributions of these two motifs, we tested two additional mutations. Previous work indicates that mutation of a Thr within the sequence (γ₆T₆29A) disrupts the effect of a dominant negative dynamin cDNA (which blocks endocytosis via clathrin-coated pits) but does not alter its regulation by Nedd4-2 (39). Conversely, mutation of the three prolines (β₆P₆16–618A) prevents ENaC regulation by Nedd4-2 but does not disrupt clathrin-mediated endocytosis (39). We tested the effects of these mutations on ENaC endocytosis using both electrophysiological and biochemical assays. Compared with wild type, γ₆T₆29A did not alter the decline in trypsin-activated current (Fig. 4A) or the removal of the 65-kDa cleaved form of αC1-2-FLAG from the cell surface (Fig. 4, B and C). In contrast, endocytosis was decreased by β₆P₆16–618A, similar to mutation of β₃Y₆20A or deletion of the β C terminus. These findings support a critical role for Nedd4-2 in mediating ENaC endocytosis. We cannot exclude a role for the YXXØ motif in regulating ENaC endocytosis in other cells.

**Nedd4-2 Increases ENaC Endocytosis**—As a more direct test of the role of Nedd4-2 in ENaC endocytosis, we transfected cells with Nedd4-2 siRNA. In previous work, we found that Nedd4-2 siRNA specifically silenced Nedd4-2 but not the related E3 ubiquitin ligase Nedd4 (26). Here we found that Nedd4-2 siRNA (compared with GFP siRNA) decreased the rate of removal of trypsin-activated channels from the cell surface in FRT epithelia, indicating a decrease in the rate of endocytosis (Fig. 5A). Likewise, Nedd4-2 siRNA reduced removal of cleaved αC1-2-FLAG from the cell surface in HEK 293T cells (Fig. 5, B and C). As a second approach, we overexpressed a dominant negative form of Nedd4-2 that contains the four WW domains that bind to ENaC but lacks the HECT domain that catalyzes ENaC ubiquitination (7). The dominant negative Nedd4-2 also decreased removal of cleaved channels from the cell surface (Fig. 5, B and C). Together, the data indicate that endogenous Nedd4-2 mediates ENaC endocytosis.

In Fig. 6, we tested the effect of Nedd4-2 overexpression on ENaC endocytosis. In HEK 293T cells, overexpression of Nedd4-2 increased endocytosis of cleaved channels (Fig. 6A). In contrast, overexpression did not alter the rate of ENaC endocytosis in FRT epithelia (Fig. 6B).
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**FIGURE 4.** Disruption of Nedd4-2 binding motif decreases ENaC endocytosis. A, FRT epithelia were co-transfected with ENaC, α, β, and γ (wild-type or P616–618A) (0.33 μg each). Plot shows trypsin-activated amiloride-sensitive current (relative to 0 min) versus time after removal of trypsin in the bathing solution (mean ± S.E., n = 8–14). B and C, HEK 293T cells transfected with αCl-2-FLAG, β (wild-type or P616–618A), and γ (wild-type or T629A) ENaC subunits (5.33 μg each) were treated with trypsin (5 μg/ml for 5 min), incubated at 37 °C for 0, 15, or 60 min, and then biotinylated. Biotinylated αCl-2-FLAG was isolated with NeutrAvidin beads and detected by immunoblot (anti-FLAG) (B), and the cleaved band (65 kDa, arrowhead) was quantitated by densitometry (C) (mean ± S.E., n = 3–8).

**FIGURE 5.** Nedd4-2 silencing decreases ENaC endocytosis. A, FRT epithelia were co-transfected with ENaC, α, β, and γ (0.27 μg each) and either Nedd4-2 siRNA or control GFP siRNA (0.2 μg). Plot shows trypsin-activated amiloride-sensitive current (relative to 0 min) versus time after removal of trypsin from the bathing solution (mean ± S.E., n = 14). B and C, HEK 293T cells were transfected with αCl-2-FLAG, β, and γ ENaC subunits (5.33 μg each) and siRNA against Nedd4-2 siRNA or GFP (4 μg) or a dominant negative Nedd4-2 (12 μg, with 1.33 μg of each ENaC subunit). The cells were treated with trypsin (5 μg/ml for 5 min), incubated at 37 °C for 0, 15, or 60 min, and then biotinylated. Biotinylated αCl-2-FLAG was isolated with NeutrAvidin beads and detected by immunoblot (anti-FLAG) (B), and the cleaved band (65 kDa, arrowhead) was quantitated by densitometry (C) (mean ± S.E., n = 3–4).

**FIGURE 6.** Nedd4-2 overexpression increases ENaC endocytosis. A, HEK 293T cells were transfected with αCl-2-FLAG, β, and γ ENaC subunits (5.27 μg each) and either Nedd4-2 cDNA or control GFP cDNA (2 μg). 48 h after transfection, the cells were treated with trypsin (5 μg/ml for 5 min), incubated for 0, 5, or 10 min at 37 °C, and then biotinylated. Biotinylated αCl-2-FLAG was isolated with NeutrAvidin beads and detected by immunoblot (anti-FLAG), and the cleaved band (65 kDa) was quantitated by densitometry (mean ± S.E., n = 3–6). B, FRT epithelia were co-transfected with α, β, and γ Cl-2 ENaC subunits (0.23 μg each) and either Nedd4-2 cDNA (+Nedd4-2) or GFP cDNA (–Nedd4-2) (0.3 μg). Plot shows trypsin-activated amiloride-sensitive current (relative to 0 min) versus time after removal of trypsin from the bathing solution (mean ± S.E., n = 4).

**DISCUSSION**

How does Nedd4-2 decrease ENaC surface expression? Our data indicate that Nedd4-2 increases the rate of ENaC endocytosis. First, we found that ENaC endocytosis was reduced by deletion or mutation of the Nedd4-2 binding sites (PY motifs) in α, β, and γ ENaC. Second, this effect was specific for the PY motif; a mutation that disrupts the YXXØ motif did not alter endocytosis. Third, endocytosis was reduced by silencing of Nedd4-2, whereas overexpression of Nedd4-2 increased endocytosis (in HEK 293T cells). Interestingly, Nedd4-2 overexpression did not alter ENaC endocytosis in FRT epi-
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FIGURE 7. Nedd4-2 increases degradation of cell surface ENaC. HEK 293T cells were transfected with α-FLAG, β, and γENaC subunits (1 μg each) and either Nedd4-2 cDNA (+Nedd4-2) or control GFP cDNA (−Nedd4-2) (0.04 μg). 48 h after transfection, the cells were biotinylated and then incubated for 0, 40, or 80 min at 37 °C. Following lysis of the cells, biotinylated α-FLAG was isolated with NeutrAvidin beads and detected by immunoblot (anti-FLAG) (A), and the cleaved band was quantitated by densitometry (mean ± S.E., n = 4) (B).

We speculate that in these cells, endogenous Nedd4-2 levels are saturated with respect to endocytosis, such that a decrease in Nedd4-2 (siRNA) reduces endocytosis but an additional increase in Nedd4-2 (overexpression) does not increase endocytosis.

In contrast to this lack of effect of Nedd4-2 overexpression on ENaC endocytosis in FRT epithelia, our previous work found that Nedd4-2 overexpression decreased ENaC current in these cells (12). This indicates that increased endocytosis is not the sole mechanism by which Nedd4-2 regulates ENaC. Consistent with this conclusion, we found that Nedd4-2 overexpression increased degradation of the cell surface fraction of ENaC. The data support a model in which Nedd4-2 regulates ENaC at two distinct sites in the endocytic pathway. First, Nedd4-2 increases ENaC endocytosis from the cell surface into endosomes. This step is rapid, occurring over 5–15 min (depending on levels of Nedd4-2). Second, once in endosomes, Nedd4-2 targets ENaC for degradation in lysosomes, decreasing the ENaC pool available for recycling back to the cell surface. This is consistent with the recent report that a Liddle syndrome mutant decreased ENaC colocalization with a lysosomal marker (31). The net effect of both is to reduce the expression of ENaC at the cell surface and, hence, decrease epithelial Na⁺ transport.

Previous studies have reported a half-life for removal of ENaC from the cell surface ranging from 30 min to 3.6 h (31, 32, 42–44). The half-life that we observed was shorter; a rapid phase of endocytosis occurred over 15 min in FRT cells and in HEK 293 cells the t½ for endocytosis was 15 min. This difference, as well as the wide variability in previous reports, could be explained by methodological differences. In each of the previous reports, the strategy was to inhibit protein synthesis (cycloheximide) or disrupt the Golgi (brefeldin A) and then measure decay of ENaC current or surface expression. These drugs have also been used to show that current decay is slowed by Liddle syndrome mutations (31, 32, 42). One limitation of this strategy is the significant and (unknown) time lag between addition of the drug and depletion of the biosynthetic pool of ENaC available for insertion into the plasma membrane. A recent study suggested that this pool was depleted very slowly following addition of cycloheximide in Madin-Darby canine kidney cells transfected with ENaC (31). This would lead to an underestimation of the endocytosis rate because the surface pool would be replenished by exocytosis of new channels. Moreover, cycloheximide and brefeldin A could inhibit synthesis/trafficking of a variety of proteins that mediate or regulate ENaC endocytosis. Rather than disrupt ENaC synthesis or trafficking, our strategy was to acutely modify a pool of channels at the cell surface (by proteolytic cleavage), which allowed us to follow the channels as they were removed from the surface using functional and biochemical assays. Somewhat analogous to a pulse-chase assay, this strategy has several advantages. Because of the discrete nature of the intervention, it provided improved time resolution compared with previous work. Moreover, the shift in molecular mass of αENaC caused by cleavage allowed us to distinguish channels undergoing endocytosis from newly synthesized channels trafficking to the cell surface. In contrast to studies using brefeldin A or cycloheximide, our approach should not alter synthesis or exocytosis of proteins involved in ENaC trafficking, increasing specificity of the assay. Finally, this strategy allowed us to selectively quantitate endocytosis of the active (proteolytically cleaved) form of ENaC.

The strategy we used also requires assumptions. First, the electrophysiological assay assumes that there are no time-dependent changes in ENaC gating (i.e., rundown); decreased current could result if P0 decreased over time. We think this is a good assumption, as we have not observed evidence of significant rundown in FRT epithelia. Moreover, the electrophysiological data were corroborated by a biochemical approach (biotinylation) that counted channel number, rather than activity. Second, the strategy assumes that channels cleaved by trypsin are endocytosed at the same rate as channels cleaved by endogenous proteases. Two observations support this assumption; trypsin and endogenous proteases generate αENaC cleavage fragments that are very similar in size, and functionally they appear to cleave an overlapping set of residues (7, 27). We do not yet know whether full-length/inactive channels are endocytosed at the same rate as cleaved/active channels. In fact, our previous work raised the interesting possibility that Nedd4-2 may selectively regulate trafficking of proteolytically cleaved channels, because it altered that ratio of cleaved to full-length channels at the cell surface (7). Additional work will be necessary to determine whether this change in ratio resulted from differences in endocytosis. Third, the strategy assumes that the rate of endocytosis measured for αENaC reflects endocytosis of functional ENaC channel complexes composed of all three subunits. In support of this assumption, we found that efficient expression of αENaC at the cell surface in HEK 293T cells requires coexpression with β and γENaC, suggesting that the three subunits primarily traffic as a heteromultimeric complex in these cells. Furthermore, the electrophysiological assay we used selectively quantitated endocytosis of functional channels; our previous work indicates that in FRT cells these channels are composed of all three ENaC subunits (30).

In FRT epithelia, ENaC endocytosis occurred over a biphasic time course. There are two potential explanations. First, there could be two or more distinct pools of ENaC with differing

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stability at the cell surface, one pool that is rapidly endocytosed and a second more stable pool. A variety of mechanisms could underlie this difference, including interactions with associated proteins that modulate endocytosis or localization in membrane microdomains with differing turnover rates. Second, the slower component could reflect recycling of endocytosed trypsin-activated channels late in the time course of the experiment, which would reduce the apparent rate of endocytosis. Our data certainly do not exclude a role for Nedd4-2 in the regulation of ENaC recycling. Although not the primary purpose of the current study, we observed a decrease in ENaC exocytosis when the channel contained a mutation in the Nedd4-2 binding sequence (PY motif, Fig. 2, A and B). However, based on trypsin sensitivity, this did not appear to arise from recycling of channels but reflected a pool of newly synthesized (trypsin-sensitive) channels. This finding is consistent with previous work from our laboratory and others (30–32).

Elucidating the mechanisms by which Nedd4-2 regulates ENaC surface expression is important for our understanding of Na$^+$ homeostasis and the pathogenesis of disease. Nedd4-2 is a critical convergence point for the regulation of epithelial Na$^+$ transport. By decreasing the expression of ENaC at the cell surface, Nedd4-2 reduces Na$^+$ reabsorption to maintain Na$^+$ homeostasis. Defects in this regulation cause Liddle syndrome, an inherited form of hypertension. Moreover, single nucleotide polymorphisms in Nedd4-2 have been linked to hypertension (45), raising the intriguing possibility that Nedd4-2 may play a role in more common forms of hypertension. Our findings, together with previous work, focus attention on the endocytic pathway for identification of new candidate genes and potential therapeutic targets for hypertension and other diseases of Na$^+$ homeostasis.

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