Nedd4-2 Catalyzes Ubiquitination and Degradation of Cell Surface ENaC

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Epithelial Na⁺ absorption is regulated by Nedd4-2, an E3 ubiquitin ligase that reduces expression of the epithelial Na⁺ channel (ENaC) at the cell surface. Defects in this regulation cause Liddle’s syndrome, an inherited form of hypertension. Previous work found that Nedd4-2 binds to ENaC via PY motifs located in the C-termini of α, β, and γENaC. However, little is known about the mechanism by which Nedd4-2 regulates ENaC surface expression. Here we found that Nedd4-2 catalyzes ubiquitination of α, β, and γENaC; Nedd4-2 overexpression increased ubiquitination whereas Nedd4-2 siRNA decreased ubiquitination. Although Nedd4-2 increased both mono/oligo-ubiquitinated and multi-ubiquitinated forms of ENaC, monoubiquitination was sufficient for Nedd4-2 to reduce ENaC expression and reduce ENaC current. Ubiquitination was disrupted by Liddle’s syndrome-associated mutations in ENaC or mutation of the catalytic HECT domain in Nedd4-2. Several findings suggest that the interaction between Nedd4-2 and ENaC is localized to the cell surface. First, Nedd4-2 bound to a population of ENaC at the cell surface. Second, Nedd4-2 catalyzed ubiquitination of cell surface ENaC. Third, Nedd4-2 selectively reduced ENaC expression at the cell surface, but did not alter the quantity of immature ENaC in the biosynthetic pathway. Finally, Nedd4-2 induced degradation of the cell surface pool of ENaC. Together, the data suggest a model in which Nedd4-2 binds to and ubiquitinates ENaC at the cell surface, which targets surface ENaC for degradation, and hence, reduces epithelial Na⁺ transport.

The epithelial Na⁺ channel (ENaC) functions in Na⁺ transport across epithelia in the kidney collecting duct and connecting tubule, lung, and distal colon, where it plays a critical role in Na⁺ homeostasis. The channel is composed of three homologous subunits (α, β, and γENaC) (reviewed in (1,2)). Mutations in β and γENaC cause Liddle’s syndrome, an inherited form of hypertension (3). Moreover, most of the known genetic causes of hypertension are caused by defects in ENaC regulation. Defective ENaC regulation may also contribute to lung disease in cystic fibrosis (4). Thus, understanding the mechanisms that regulate ENaC may provide new insights into the pathogenesis of hypertension, cystic fibrosis, and other diseases of Na⁺ homeostasis.

ENaC is regulated in large part by mechanisms that control its expression at the apical membrane of epithelia. Several findings have implicated an important role for Nedd4-2, an E3 ubiquitin-protein ligase. First, Nedd4-2 and ENaC interact through the binding of PY motifs (PPxYxxL) located in the C-termini of α, β, and γENaC to multiple WW domains in Nedd4-2 (5). Importantly, ENaC mutations that disrupt this interaction cause Liddle’s syndrome by increasing ENaC surface expression (6-8). Second, Nedd4-2 overexpression decreases ENaC current by reducing its expression at the cell surface (8,9). Third, silencing of endogenous Nedd4-2 by RNA interference increases ENaC current (10). Finally, aldosterone and vasopressin regulate ENaC in part by inducing phosphorylation of Nedd4-2 (via serum and glucocorticoid-induced kinase and PKA, respectively), which decreases Nedd4-2 binding to ENaC (9,11,12).

However, critical questions remain about the mechanism by which Nedd4-2 regulates ENaC. First, it is not known if Nedd4-2 regulates ENaC directly by catalyzing ubiquitination of one or more ENaC subunit, or indirectly by catalyzing
ubiquitination of an accessory protein. Staub, et al. reported that α and γENaC are substrates for ubiquitination, and that mutation of lysines at the N-termini of these subunits increased ENaC surface expression (13). More recent work suggests that βENaC might also be a substrate for ubiquitination (14,15). However, it is not known if Nedd4-2 or other ubiquitin ligases catalyze ENaC ubiquitination. Second, the cellular location at which Nedd4-2 binds to and regulates ENaC has not been identified. Nedd4-2 could interact with ENaC in the biosynthetic pathway and block its trafficking to the cell surface. Alternatively, Nedd4-2 could interact with ENaC at the cell surface and increase its endocytosis and/or degradation. In this work, our goal was to test the hypothesis that Nedd4-2 binds to ENaC and catalyzes its ubiquitination at the cell surface, which targets this pool of channels for degradation.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Human α, β, and γENaC (16,17), Nedd4-2 (11), and Nedd4-2C821A (18) in pMT3 were cloned as described previously. α-FLAG, β-FLAG, and γ-FLAG were generated by insertion of a FLAG epitope (DYKDDDDK) at the C-terminus. PY motif mutations were generated in αENaC (Y644A), βENaC (R566X and Y620A), and γENaC (Y627A) as described previously (6). Nedd4-2WW1-4 was generated by mutating two residues in each of the four WW domains (WW1, V210W and H212G; WW2, V367W and H369G; WW3, I440W and H442G; WW4, I492W and H494G). These mutations abolish Nedd4-2 binding to ENaC, similar to previous work with Nedd4 (19). HA tagged ubiquitin (in pMT123) was provided by Dirk Bohmann (University of Rochester), and lysine-less ubiquitin cDNA (Ub-0K) by Joan Conaway (Stowers Institute for Medical Research).

Ubiquitination Assay—To detect ENaC ubiquitination, HEK 293T cells were transfected with or without cDNAs encoding ubiquitin-HA and α, β, and γENaC (one subunit contained FLAG epitope) using Lipofectamine 2000 (Invitrogen). In some studies, cells were cotransfected with Nedd4-2 (wild-type or C821A) or green fluorescent protein (GFP, control) cDNA, or with siRNA against Nedd4-2 (10) or GFP (control). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10 µM amiloride. To inhibit proteasomal degradation, 10 µM N-acetyl-Leu-Leu-norleucinal (ALLN) was added to some cells 2 h before lysis. 24 h after transfection, cells were solubilized in lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% Triton X-100, and protease inhibitor cocktail (Sigma)), and 800 µg of protein was immunoprecipitated with anti-HA antibody (1:300, Sigma) and immobilized protein A (Pierce). After extensive washing, immunoprecipitated (ubiquitinated) proteins were separated by SDS-PAGE, detected by immunoblot with anti-FLAG M2 monoclonal antibody-peroxidase conjugate (1:5000, Sigma), and quantitated by densitometry.

Cell Surface Biotinylation and Protein Interactions—HEK 293T cells were transfected with α, β, and γENaC (1 µg each) and Nedd4-2-HA (0.04 µg). 24 h later, the cells were washed with PBS-CM (PBS with 1 mM MgCl2 and CaCl2), cell surface proteins were labeled with 0.5 mg/ml Sulfo-NHS-biotin (Pierce) in PBS-CM for 30 min on ice, then cells were quenched with 100 mM glycine in PBS-CM for 10 min on ice. After washing three times with PBS-CM, cells were lysed in NP-40 lysis buffer (0.4% sodium deoxycholate, 1% NP-40, 63 mM EDTA, 50 mM Tris-HCl (pH 8), and protease inhibitor cocktail). Biotinylated (cell surface) and interacting proteins were isolated by incubating cell lysate with immobilized NeutrAvidin beads (Pierce) for 12 h at 4 °C. Following separation by SDS-PAGE, biotinylated αENaC and coprecipitated Nedd4-2 were detected by immunoblot. In some experiments, unbiotinylated ENaC subunits were immunoprecipitated from either the NeutrAvidin supernatant or from the total cellular lysate.

Cell Surface Ubiquitination—HEK 293T cells transfected with or without α, β-FLAG, and γENaC, Nedd4-2, and ubiquitin-HA were biotinylated at 4 °C (to prevent protein trafficking) as above, or not biotinylated as control. Following solubilization in 1% Triton X-100 lysis buffer, βENaC was immunoprecipitated (anti-FLAG M2 affinity gel) from 800 µg of cell lysate. βENaC was eluted from the gel by incubating with SDS-PAGE sample buffer (100 mM dithiothreitol, 20%
glycerol, 100 mM Tris-Cl, pH 6.8, and 4% SDS) at 95 °C for 5 min. The supernatant was diluted with 11 volumes of lysis buffer and biotinylated βENaC was isolated by incubation with immobilized NeutrAvidin beads and separated by SDS-PAGE. Ubiquitinated βENaC at the cell surface was detected by immunoblot (anti-HA antibody, 1:2000).

Degradation of Cell Surface ENaC—HEK 293T cells were transfected with α, β-FLAG and γENaC (wild-type or αY644A, βY620A, γY627A) with Nedd4-2 or GFP. 24 h after transfection, cells were biotinylated on ice for 30 min and then incubated at 37 °C for 0, 40, 80, 120 min. Cell surface βENaC was isolated by incubation with immobilized NeutrAvidin beads, detected by immunoblot with anti-FLAG M2 monoclonal antibody-peroxidase conjugate, and quantitated by densitometry.

Short-Circuit Current—Fischer rat thyroid (FRT) cells on permeable filter supports were cotransfected with α, β, and γENaC (0.17 µg each) with or without Nedd4-2 (0.4 µg) and Ub-0K (0.5 µg) using TFX50 (Promega), as described previously (11); total cDNA was held constant with GFP cDNA. Short-circuit Na⁺ current was measured using modified Ussing chambers (Warner Instrument Corporation). 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.

RESULTS

Nedd4-2 Catalyzes ENaC Ubiquitination—To determine which ENaC subunits are substrates for ubiquitination, we cotransfected HEK 293T cells with α, β, and γENaC (0.17 µg each) with or without Nedd4-2 (0.4 µg) and Ub-0K (0.5 µg) using TFX50 (Promega), as described previously (11); total cDNA was held constant with GFP cDNA. Short-circuit Na⁺ current was measured using modified Ussing chambers (Warner Instrument Corporation). 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.

ENaC subunits were isolated by immunoprecipitation of ubiquitin (anti-HA) and detected by immunoblot (anti-FLAG). We detected ubiquitinated α, β, and γENaC in cells cotransfected with Ub-HA, but not in cells transfected separately with either ENaC or Ub-HA (Fig. 1, top). Interestingly, we detected at least two different ubiquitinated forms of each subunit; faster migrating bands likely represent ENaC subunits with one or a few ubiquitins attached (monoubiquitinated or oligoubiquitinated), whereas slower migrating bands are multi-ubiquitinated (polyubiquitinated or poly-monoubiquitinated) ENaC.

To test if Nedd4-2 catalyzes ubiquitination of one or more ENaC subunit, we used two strategies. First, we overexpressed Nedd4-2. In these experiments, ALLN was omitted to reduce basal levels of ubiquitination. Fig. 2A-C shows representative immunoblots and Fig. 2D-F shows quantitation of multi-ubiquitinated (left panel) and mono/oligoubiquitinated ENaC subunits (right panel) Nedd4-2 significantly increased ubiquitination of α, β, and γENaC (compare lanes 1 and 2 in Fig. 2A-C). Moreover, Nedd4-2 increased both the faster and slower migrating forms. As controls for specificity, we did not detect ubiquitinated proteins in cells lacking ENaC (Fig. 2A and 2C, lanes 5 and 7, and Fig. 2B, lane 7) or in cells expressing ENaC without Ub-HA and Nedd4-2 (lane 6).

As a second strategy, we silenced expression of endogenous Nedd4-2 with Nedd4-2 siRNA. We characterized this siRNA in previous work; it selectively decreased Nedd4-2 protein levels (but not the related E3 ligase Nedd4), and it increased ENaC current in epithelia (10). Here we found that Nedd4-2 siRNA decreased ubiquitination of αENaC (Fig. 3A and 3B).

Nedd4-2 HECT Domain and ENaC PY Motifs are Required for ENaC Ubiquitination—The C-terminus of Nedd4-2 contains a HECT domain. Through the binding of ubiquitin to Cys-821, this domain catalyzes ubiquitination of target proteins. Previous work indicates that the HECT domain is required for Nedd4-2 to inhibit ENaC (5,20). Mutation of Cys-821 to Ala abolished Nedd4-2-mediated ubiquitination of α and β ENaC, as well as the slower migrating form of γENaC (Fig. 2A-C,
compare lanes 2 and 3, quantified in Fig. 2D-F). Interestingly, the mutant Nedd4-2 increased the faster migrating mono- or oligoubiquitinated form of γENaC (compared to the group without Nedd4-2), although much less than wild-type Nedd4-2 (Fig. 2C and 2F). Thus, the catalytic activity of the HECT domain is required for Nedd4-2 to induce ubiquitination of α, β, and the slower migrating form of γENaC, but not mono/oligoubiquitinated γENaC.

Nedd4-2 binds to ENaC via PY motifs located in the cytoplasmic C-terminus of each ENaC subunit. Mutation of these motifs prevents Nedd4-2 from inhibiting ENaC (5). To test if binding is required for ubiquitination, we disrupted the PY motifs. Deletion of this motif in ENaC by a Liddle’s syndrome mutation (R566X, disrupted the PY motifs. Deletion of this motif in ENaC is required for ubiquitination, we Nedd4-2 from inhibiting ENaC (5). To test if ENaC subunit. Mutation of these motifs prevents located in the cytoplasmic C-terminus of each mono/oligoubiquitinated migrating form of ENaC. However, the slower migrating form of ubiquitinated βENaC (that did not reach statistical significance), but not the slower migrating form (Fig. 2B lane 4 and Fig. 2E). Mutation of the PY motif (Y620A, “βγY,”) produced a minimal decrease in the faster migrating form of ubiquitinated βENaC (Figs. 2A and 2C, compare lanes 2 and 4, quantitated in 2D and 2F). Mutation of the PY motif (Y620A, “βγY”) produced a minimal decrease in the faster migrating form of ubiquitinated βENaC (that did not reach statistical significance), but not the slower migrating form (Fig. 2B lane 4 and Fig. 2E). However, simultaneous mutation of the PY motifs in α, β, and γENaC abolished ubiquitination of βENaC (Fig. 2B and 2E). Taken together, these data suggest that Nedd4-2 binds to the PY motifs of ENaC subunits, then catalyzes ubiquitination via the HECT domain.

Nedd4-2 and ENaC Interact at the Cell Surface — Although it is clear from previous work that Nedd4-2 binds to ENaC, the cellular location where this interaction occurs has not been identified. We hypothesized that Nedd4-2 interacts with ENaC at the cell surface. To test this hypothesis, we transfected HEK 293T cells with ENaC and Nedd4-2 (containing an HA epitope). Cell surface proteins were then biotinylated and isolated with NeutrAvidin beads. In this fraction of cell surface biotinylated proteins, we detected (by immunoblot) full length (90 kDa) and proteolytically cleaved (65 kDa) forms of αENaC (Fig. 4A, bottom panel). Nedd4-2 was also present in this cell surface fraction in cells expressing ENaC, but not in cells lacking ENaC (Fig. 4A, top panel). These results indicate that Nedd4-2 binds to ENaC at the cell surface. Moreover, they show that Nedd4-2 is not itself a substrate for biotinylation, consistent with its intracellular location.

To test if Nedd4-2 ubiquitinates ENaC at the cell surface, we subjected cells to cell surface biotinylation, followed by sequential precipitation with anti-FLAG beads (to isolate ENaC), then NeutrAvidin beads (to isolate cell surface ENaC). We then analyzed the ubiquitination state of cell surface ENaC by immunoblot for Ub-HA. Fig. 4B shows that βENaC was highly ubiquitinated in the presence but not in the absence of Nedd4-2. Together the data suggest that Nedd4-2 binds to and ubiquitinates ENaC at the cell surface.

Nedd4-2 Selectively Decreases Steady-State Levels of ENaC at the Cell Surface — If Nedd4-2 regulates ENaC at the cell surface, we predict that it should decrease steady state levels of ENaC at the cell surface, but have little effect on intracellular ENaC (which primarily reflects immature ENaC in the biosynthetic pathway). To test this prediction, we biotinylated cell surface proteins in cells transfected with ENaC (α, β, γ) and Nedd4-2 (0-0.5 μg). We separated biotinylated (surface) proteins from non-biotinylated (intracellular) proteins by binding to NeutrAvidin beads, then detected βENaC in each fraction by immunoblotting; Fig. 5A shows representative immunoblots and Fig. 5B shows quantitation of the Nedd4-2 dose-response relationship. Nedd4-2 did not alter levels of intracellular βENaC (Fig. 5A and 5B, top panels). In contrast, Nedd4-2 produced a dose-dependent decrease in βENaC at the cell surface (Fig. 5A and 5B, bottom panels). Thus, Nedd4-2 selectively regulates ENaC at the cell surface.

To test if binding is required for Nedd4-2 to reduce ENaC surface expression, we used two strategies. First, we mutated the PY motif in βENaC (βY620A). In the absence of Nedd4-2, this mutation increased ENaC surface expression (Fig. 5A, bottom panel), consistent with previous work (6,8). Moreover, it reduced the effect of Nedd4-2 on ENaC surface expression (Fig. 5A and 5B, bottom panels). In contrast, this mutation had little effect on levels of intracellular ENaC (Fig. 5A and 5B, top panels). As a second strategy, we mutated the four Nedd4-2 WW domains (WW domains 2-4 mediate binding to ENaC). The mutant Nedd4-2 failed to decrease ENaC surface expression (Fig. 5C). The ubiquitin ligase activity.
of Nedd4-2 was also required; mutation of the HECT domain (C821A) prevented Nedd4-2 from decreasing ENaC surface expression (Fig. 5D).

**Nedd4-2 Induces Degradation of the Cell Surface Pool of ENaC**—To further test the hypothesis that Nedd4-2 selectively regulates ENaC at the cell surface, we asked whether Nedd4-2 induces degradation of channels that have reached the cell surface. We selectively measured the rate of degradation of the cell surface pool of ENaC. This pool was labeled by biotinylation (at 4 °C to prevent trafficking), then cells were warmed to 37 °C for 0-120 min to allow endocytosis and degradation of biotinylated ENaC. In the absence of Nedd4-2, there was a time-dependent loss of biotinylated βENaC (Fig. 6A and 6B); the half-life of degradation was approximately one hour. Nedd4-2 dramatically increased the rate of degradation of cell surface ENaC, shortening the half-life to < 20 min (Fig. 6A and 6B). Mutation of the PY motifs in α, β, and γENaC abolished the effect of Nedd4-2 (Fig. 6A and 6C). Thus, once ENaC reaches the cell surface, Nedd4-2 induces its degradation. This negative regulation is disrupted in Liddle’s syndrome.

**ENaC Monoubiquitination is Sufficient for Nedd4-2-induced Degradation**—Data in Fig. 2 suggest that Nedd4-2 catalyzes both monoubiquitination and polyubiquitination of α, β, and γENaC. To assess the relative functional importance of these two species, we used a mutant ubiquitin; mutation of each lysine (Ub-0K) prevents formation of polyubiquitin chains (21,22). Expression of Ub-0K increased levels of βENaC at the cell surface (Fig. 7A, compare lanes 1 and 5). This suggests that ENaC surface expression is in part controlled by polyubiquitination. However, Ub-0K did not prevent Nedd4-2 from decreasing ENaC surface expression; the Nedd4-2 dose-response relationship was identical in the presence or absence of Ub-0K (when each group was normalized to surface expression in the absence of Nedd4-2) (Fig. 7A and 7B). In Fischer rat thyroid epithelia transfected with α, β, and γENaC, Ub-0K did not prevent Nedd4-2 from decreasing ENaC current (Fig. 7C). Together the data suggest that monoubiquitination is sufficient for Nedd4-2 to regulate ENaC.

**DISCUSSION**

From previous work, it is clear that Nedd4-2 plays a key role in regulating epithelial Na+ transport. By decreasing the expression of ENaC at the cell surface, Nedd4-2 reduces renal Na+ absorption, which is critical in the maintenance of Na+ homeostasis. Defects in this regulation cause Liddle’s syndrome and may contribute to more common forms of hypertension.

Does Nedd4-2 regulate ENaC surface expression by ubiquitinating one or more ENaC subunit(s)? Nedd4-2 is an E3 ubiquitin-protein ligase. Moreover, its catalytic HECT domain is required for ENaC regulation. Thus, it seems likely that Nedd4-2 regulates ENaC by catalyzing ubiquitination of one or more substrates. These substrates could either be ENaC subunit(s) themselves, or a trans-acting protein that modulates ENaC surface expression. In support of the first possibility, we found that Nedd4-2 catalyzes ubiquitination of α, β, and γENaC. We cannot exclude the possibility that ubiquitination of a trans-acting protein by Nedd4-2 also contributes to regulation of ENaC surface expression. For example, we previously found that Nedd4-2 ubiquitinates and induces degradation of SGK, a Ser/Thr kinase that stimulates ENaC (18). In this regard, it is also intriguing that a yeast homologue of Nedd4-2 (Rsp5) induces endocytosis of Ste2p by catalyzing ubiquitination of a component of the endocytosis machinery (23).

Does Nedd4-2 catalyze monoubiquitination or polyubiquitination of ENaC? Our data suggest that Nedd4-2 does both. Nedd4-2 increased the quantity of faster migrating ubiquitinated forms of α, β, and γENaC. Based on the relative molecular mass, this form is consistent with subunits containing one or a small number of ubiquitins (mono- or oligoubiquitinated). Nedd4-2 also increased a high molecular mass smear, consistent with polyubiquitin chains. However, because the N-terminus of each ENaC subunit contains multiple lysines, attachment of a single ubiquitin to multiple residues (polymonoubiquitination) could also contribute to the higher molecular mass forms. In addition to ENaC ubiquitination induced by overexpression of
Nedd4-2, we also observed ubiquitinated α, β, and γENaC in cells not transfected with Nedd4-2. This is consistent with previous work from other labs (although there was disagreement about whether βENaC was a substrate for ubiquitination) (13-15). Our data suggest that endogenous Nedd4-2 contributes to this basal level of ubiquitination; silencing of Nedd4-2 reduced ENaC ubiquitination. Importantly, ENaC regulation by Nedd4-2 was intact under conditions that prevented polyubiquitination (Ub-0K). Thus, although Nedd4-2 catalyzes both mono- and polyubiquitination of ENaC, monoubiquitination is sufficient for Nedd4-2 to induce ENaC degradation (conversely, polyubiquitination is not necessary).

E3 ligases function at a variety of cellular locations. For example, gp78 and CHIP participate in quality control in the biosynthetic pathway, targeting misfolded proteins in the endoplasmic reticulum for degradation in the proteasome (24,25). Other E3 ligases (e.g. Rsp5, Mdm2) target membrane proteins for endocytosis and degradation (23,26). Previous work localized Nedd4-2 both in the cytoplasm and at the cell surface (27). Moreover, the C2 domain (required for Ca2+-induced localization to the cell surface (27)) is not required for Nedd4-2 or the related E3 ligase Nedd4 to reduce ENaC surface expression (11,19,28). These observations raised the possibility that Nedd4-2 and ENaC interact at an intracellular location (27). Our current data suggest an alternative model in which Nedd4-2 binds to ENaC and catalyzes its ubiquitination at the cell surface. First, we found that Nedd4-2 bound to ENaC at the cell surface. Second, Nedd4-2 catalyzed ubiquitination of cell surface ENaC. Third, Nedd4-2 decreased ENaC expression at the cell surface, but had no effect on intracellular ENaC, which principally reflects immature channels in the biosynthetic pathway. Finally, following ENaC trafficking to the cell surface, Nedd4-2 induced its degradation. However, we cannot exclude an additional role for Nedd4-2 at a cytoplasmic location, either in the biosynthetic or endosomal sorting pathways. Moreover, it seems likely that additional E3 ligases might also regulate ENaC in these locations. Consistent with this notion, silencing of Nedd4-2 did not abolish ENaC ubiquitination. Moreover, disruption of polyubiquitination (Ub-0K) increased ENaC surface expression in spite of its lack of effect on ENaC regulation by Nedd4-2. One caveat is that our studies were carried out in heterologous cell systems; additional work will be required to determine if the same mechanisms are operative in native epithelia.

We found that Liddle’s syndrome mutations disrupted Nedd4-2-mediated ubiquitination of ENaC. Because these mutations delete or alter PY motifs that mediate ENaC binding to Nedd4-2, this suggests that binding facilitates ubiquitination. Moreover, it provides a mechanistic link between the disease-causing mutations and the excessive Na+ reabsorption that leads to hypertension in patients with Liddle’s syndrome. By disrupting ubiquitination, Liddle’s syndrome mutations increase ENaC surface expression, causing excessive renal Na+ absorption. Nedd4-2 is regulated by the renin-angiotensin-aldosterone pathway. Moreover, defects in this pathway are responsible for nearly all of the known inherited forms of hypertension. Thus, understanding the mechanisms that mediate ENaC ubiquitination may help unravel the pathogenesis of more common forms of hypertension.

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FOOTNOTES

1The abbreviation used are: ENaC, epithelial Na⁺ channel; siRNA, small interfering RNA; GFP, green fluorescent protein; ALLN, N-acetyl-Leu-Leu-norleucinal; FRT, Fischer rat thyroid.
**FIGURE LEGENDS**

**Figure 1. ENaC ubiquitination.** HEK 293T cells were transfected with α, β, and γENaC (1 µg each, one subunit contained C-terminal FLAG) with or without ubiquitin-HA (3 µg) and incubated for 24 h prior to lysis (10 µM ALLN was present for the final 2 h). Ubiquitinated (top panel) and total (bottom panel) ENaC subunits were detected by immunoprecipitation followed by immunoblot, as indicated. Data are representative of at least three experiments.

**Figure 2. Nedd4-2 catalyzes ENaC ubiquitination.** HEK 293T cells were transfected with α, β, and γENaC (indicated subunit contained FLAG, 1 µg each). ENaC subunits were wild-type (“wt”) or contained the following mutations; βR566X (“βL”), βY620A (“βY-A”), αY644A (“αY-A”), and γY627A (“γY-A”). The cells were cotransfected with or without Nedd4-2 (wild-type or C821A (“H”), 2 µg) and ubiquitin-HA (3 µg), as indicated. Total transfected DNA was held constant using GFP cDNA. Ubiquitinated ENaC was detected by immunoprecipitation of ubiquitin (anti-HA) followed by immunoblot for α, β, or γENaC (anti-FLAG). A-C, representative immunoblots; D-F, quantitation of multi- and mono/oligoubiquitinated ENaC subunits by densitometry relative to wild-type ENaC + Nedd4-2 groups- lane 2 (mean ± SEM, n = 3; * p < 0.05 vs. ENaC + Nedd4-2 by t-test).

**Figure 3. Endogenous Nedd4-2 ubiquitinates ENaC.** HEK 293T cells were transfected with ENaC (α-FLAG, β, and γ, 0.8 µg each), ubiquitin-HA (2.4 µg), and siRNA targeting Nedd4-2 or GFP (“C”) (1.6 µg). Ubiquitinated αENaC was detected by immunoprecipitation of ubiquitin (anti-HA) followed by immunoblot (anti-FLAG). A, representative immunoblots; B, quantitation by densitometry (mean ± SEM, n = 3; * p < 0.005 by t-test).

**Figure 4. Nedd4-2 binds to and ubiquitinates ENaC at the cell surface.** A, HEK 293T cells were transfected with or without α-FLAG, β, and γENaC (1 µg each) and Nedd4-2-HA (0.04 µg). Cell surface proteins were biotinylated, isolated with NeutrAvidin beads, then immunoblotted with anti-HA to detect Nedd4-2 (top panel) or anti-FLAG to detect αENaC (bottom panel). B, HEK 293T cells were transfected with or without α, β-FLAG, and γENaC (1 µg each), Nedd4-2 (2 µg), and ubiquitin-HA (3 µg). Cell surface proteins were biotinylated or not biotinylated at 4 °C, as indicated. Ubiquitinated βENaC at the cell surface was isolated by immunoprecipitation (anti-FLAG) followed by binding to NeutrAvidin beads, then detected by immunoblot (anti-HA). Data are representative of at least three experiments.

**Figure 5. Nedd4-2 selectively decreases ENaC surface expression.** HEK 293T cells were transfected with α, β-FLAG (wild-type or Y620A), and γENaC (1 µg each) and Nedd4-2 (0-0.5 µg). Nedd4-2 was wild-type (A) or contained mutations in the four WW domains (C, “Nedd4-2WW”) or the HECT domain (D, “Nedd4-2C821A”). “Control” was transfected with GFP. Cell surface proteins were biotinylated, then isolated by binding to NeutrAvidin beads. Unbiotinylated proteins in the supernatant were subjected to immunoprecipitation (anti-FLAG). βENaC in the biotinylated (“surface”) and unbiotinylated (“intracellular”) fractions were detected by immunoblot (anti-FLAG). A, C, and D, representative immunoblots; B, quantitation of βENaC in the intracellular (top panel) and cell surface (bottom panel) fractions by densitometry (relative to 0 Nedd4-2 groups, n = 4). In C and D, total βENaC was detected by immunoprecipitation of cell lysates, followed by immunoblot (anti-FLAG). Data are representative of at least three experiments.

**Figure 6. Nedd4-2 induces degradation of the cell surface pool of ENaC.** HEK 293T cells were transfected with α, β-FLAG and γENaC (wild type or Y-A mutants, 1 µg each) with Nedd4-2 or GFP (0.04 µg), as indicated. Cell surface proteins were labeled by biotinylation at 4 °C, then incubated at 37
˚C for 0-120 min. Biotinylated βENaC from 500 µg (upper two panels) and 50 µg (lower panel) of cell lysates was isolated with neutravidin beads and detected by immunoblot (A). B and C, quantitation of the time-dependent loss of biotinylated βENaC relative to 0 min (n = 3).

**Figure 7. ENaC monoubiquitination is sufficient for Nedd4-2-induced degradation.** A and B, HEK 293T cells were transfected with ENaC (α, β-FLAG, γ, 1µg each), Nedd4-2 (0-0.5 µg) with or without lysine-less ubiquitin (Ub-0K, 2.5µg). Biotinylated βENaC was detected by immunoblot (A) and quantitated by densitometry relative to 0 Nedd4-2 groups (B) (n = 4). C, amiloride-sensitive short-circuit current in FRT epithelia expressing ENaC (α, β, and γ) (0.17 µg each) with or without Nedd4-2 (0.4 µg) and Ub-0K (0.5 µg)(n = 19-24).
Fig. 1
Fig. 2
Fig. 3
**Fig. 4**

**A**

| αFβγ ENaC | + | + | - |
| Nedd4-2 | - | + | + |

Pulldown: NeutrAvidin  
IB: Nedd4-2  
IB: αENaC

**B**

| αβFγ ENaC | + | + | + | - | + |
| Ub-HA | + | + | + | + | - |
| Nedd4-2 | - | - | + | - | - |
| Biotinylated | - | + | + | + | + |

IP: βENaC  
Pulldown: NeutrAvidin  
IB: Ub

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[Image 84x153 to 534x657]
Fig. 5
**Fig. 6**

(A) Time (min) 0 40 80 120

- ENaC<sub>WT</sub> + GFP
- ENaC<sub>WT</sub> + N4-2
- ENaC<sub>Y-A</sub> + N4-2

(B) β<sub>F</sub>ENaC (Rel.) 0.5

- - N4-2
- + N4-2

(C) β<sub>F</sub>ENaC (Rel.) 0.5

- Y-A + N4-2
- WT + N4-2

Time (min) 0 40 80 120
Fig. 7
Nedd4-2 catalyzes ubiquitination and degradation of cell surface ENaC
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