Relative Contribution of Nedd4 and Nedd4-2 to ENaC Regulation in Epithelia Determined by RNA Interference*

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Epithelial Na⁺ transport is regulated in large part by mechanisms that control expression of the epithelial Na⁺ channel (ENaC) at the cell surface. Nedd4 and Nedd4-2 are candidates to control ENaC surface expression, but it is not known which of these proteins contributes to ENaC regulation in epithelia. To address this question, we used RNA interference to selectively reduce expression of Nedd4 or Nedd4-2. We found that endogenous Nedd4-2, but not Nedd4, negatively regulates ENaC in two epithelial cell lines (Fischer rat thyroid and H441); small interfering RNA (siRNA) against Nedd4-2 increased amiloride-sensitive Na⁺ current (compared with control siRNA), but Nedd4 siRNA did not. A mutation associated with Liddle’s syndrome (β²ASOS) abolished the effect of Nedd4-2 siRNA, suggesting that a defect in ENaC regulation by Nedd4-2 contributes to the pathogenesis of this inherited form of hypertension. Previous work found that Nedd4-2 is phosphorylated by serum and glucocorticoid-regulated kinase, a Ser/Thr kinase induced by steroid hormones. Here we found that Nedd4-2 phosphorylation contributes to ENaC regulation by steroid hormones. Consistent with this model, ENaC stimulation by dexamethasone was reduced by Nedd4-2 siRNA and by overexpression of a mutant Nedd4-2 lacking serum and glucocorticoid-regulated kinase phosphorylation sites. Thus, endogenous Nedd4-2 negatively regulates ENaC in epithelia and is a component of a signaling pathway by which steroid hormones regulate ENaC. Defects in this regulation may contribute to the pathogenesis of hypertension.

The rate of Na⁺ transport across a variety of epithelia (e.g. kidney-collecting duct, airway, colon) is regulated in large part by mechanisms that control expression of the epithelial Na⁺ channel (ENaC) at the apical cell surface. ENaC, a heteromultimeric complex of three homologous subunits (α-, β-, and γ-ENaC), forms a pathway for Na⁺ to enter the cell at the apical membrane followed by Na⁺ exit at the basolateral membrane mediated by the Na⁺-K⁺-ATPase (1, 2). Defects in ENaC regulation cause blood pressure abnormalities (hypertension or hypotension) (3) and contribute to the pathogenesis of cystic fibrosis (4).

Although regulation of ENaC surface expression is critical for Na⁺ homeostasis, little is known about the mechanisms responsible. Previous work (reviewed in Ref. 5) identified Nedd4 and Nedd4-2, HECT WW domain ubiquitin-protein ligases, as candidates. First, Nedd4 and Nedd4-2 are capable of binding to ENaC; multiple WW domains bind to PY motifs located in the C terminus of each ENaC subunit (6–10). Importantly, mutation of these PY motifs causes an inherited form of hypertension (Liddle’s syndrome) (11, 12). Second, Nedd4 and Nedd4-2 inhibit ENaC when coexpressed in heterologous cells; a ubiquitin ligase domain catalyzes ENaC ubiquitination, targeting the channel for degradation (9, 13, 14). As a result, reduced ENaC surface expression decreases Na⁺ current. However, an important limitation is that previous studies tested the effect of Nedd4 and Nedd4-2 overexpression. Although both have the potential to inhibit ENaC, a key unanswered question is, which of these ubiquitin-protein ligases is a physiological regulator of ENaC?

ENaC surface expression is also regulated by steroid hormones; mineralocorticoids and glucocorticoids increase epithelial Na⁺ absorption primarily by increasing ENaC expression at the cell surface (reviewed in Ref. 15). Recent work suggests a potential role for Nedd4-2. Our lab and others found that Nedd4-2 is phosphorylated by serum and glucocorticoid-regulated kinase (SGK) (16, 17), a key mediator of ENaC regulation downstream of steroid hormones (18–21). Phosphorylation decreases Nedd4-2 binding to ENaC and reduces Nedd4-2 inhibition of ENaC (16, 17). This suggests the possibility that steroid hormones regulate Na⁺ transport in part through negative regulation of Nedd4-2.

In this work, we tested the hypothesis that Nedd4 and Nedd4-2 contribute to ENaC regulation in epithelia. Our strategy was to deplete cells of these proteins using RNA interference and to disrupt their function using a mutant construct. Our first goal was to distinguish the relative contribution of endogenous Nedd4 and Nedd4-2 to ENaC regulation under basal conditions and to test whether this regulation is disrupted in Liddle’s syndrome. Our second goal was to test the hypothesis that Nedd4-2 is part of a signaling pathway that links steroid hormones to ENaC.

EXPERIMENTAL PROCEDURES

RNA and cDNA Constructs—Small interfering RNAs (siRNA) were generated (Qiagen-Xeragon) against Nedd4 (5'-TAGAGGCTCGGT-GGTTGTTTTG-3') and Nedd4-2 (5'-AACCACAAAGATGTCACA-CAG-3'). These sequences correspond to human nucleotide positions 503–525 and 1103–1125, respectively. We chose sequences that are identical in human and rat but do not match other sequences in GenBank™. We tested additional siRNAs against Nedd4 and Nedd4-2 that did not decrease protein levels. As a negative control, we generated an
siRNA against green fluorescent protein (GFP, 5′-GCAAGCAGCTGGA-
"TCAACATACA-3′).

Human α-, β-, and γ-ENaC (22, 23, Nedd4 (8), Nedd4-2 (16), and
SGK (16) were cloned as described previously and subcloned into pM73.
Nedd4-GFP and Nedd4-2-GFP fusion constructs were generated by
cloning Nedd4 and Nedd4-2 in-frame to the C terminus of GFP in
pEGFP-C1 (Clontech). Nedd4-2R-α was generated by mutating Ser221,
Thr246, and Ser227 simultaneously to alanine (QuickChange, Stratagene).
SGK was generated as described by Snyder et al. (16).

Cell Culture and Transfection—Fisher rat thyroid (FRT) cells were
seeded onto permeable filter supports (Millicell PCF, 0.4
μm pore size, 12 mm diameter) and maintained in F-12 Coon’s medium (Sigma) with
5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, as described previously (24). One day after
seeding, cells were cotransfected with siRNA and cDNAs encoding
Nedd4, Nedd4-2, Nedd4-GFP, Nedd4-2-GFP, or α-, β-, and γ-ENaC. In
experiments where Nedd4 or Nedd4-2 cDNA was varied, total DNA was
kept constant with GFP cDNA (expression of GFP did not alter ENaC
Na+ currents). siRNA (0.5 ng/μl) and cDNA (5 ng/μl) were mixed with
TFX 50 (Promega, 40 ng/μl) in 200 μl/Millicell serum-free F-12 Coon’s
medium for 15 min and then transferred to the apical surface of the
monolayer. One h later, the apical solution was supplemented with 200
μl of F-12 Coon’s medium containing fetal calf serum (final concentra-
tion 5%). After 24 h, the cells were incubated in serum-free medium for
18 h and then in serum-free medium with or without dexamethasone
(100 ng/ml) for 6 h.

H441 cells (American Type Culture Collection, Manassas, VA) were
grown on permeable filter supports in RPMI 1640 medium with 8.5%
 fetal calf serum, 5 μg/ml transferrin, 5 μg/ml selenium, 100 nM
ferritin, 5 μg/ml streptomycin, 50 μg/ml penicillin, 100 μg/ml strepto-
mycin at 37 °C, as described previously (24). One day after
seeding, cells were cotransfected with siRNA and cDNAs encoding
Nedd4, Nedd4-2, Nedd4-GFP, Nedd4-2-GFP, or α-, β-, and γ-ENaC. In
experiments where Nedd4 or Nedd4-2 cDNA was varied, total DNA was
kept constant with GFP cDNA (expression of GFP did not alter ENaC
Na+ currents). siRNA (0.5 ng/μl) and cDNA (5 ng/μl) were mixed with
TFX 50 (Promega, 40 ng/μl) in 200 μl/Millicell serum-free F-12 Coon’s
medium for 15 min and then transferred to the apical surface of the
monolayer. One h later, the apical solution was supplemented with 200
μl of F-12 Coon’s medium containing fetal calf serum (final concentra-
tion 5%). After 24 h, the cells were incubated in serum-free medium for
18 h and then in serum-free medium with or without dexamethasone
(100 ng/ml) for 6 h.

H441 cells were transfected with Nedd4-GFP or Nedd4-2-GFP with or
without siRNA (Nedd4 or Nedd4-2) as described above. The cells were
fixed with 4% paraformaldehyde 48 h after transfection. Nedd4 and
Nedd4-2 proteins were detected as GFP fluorescence using confocal
microscopy (Bio-Rad MRC 1024, krypton/argon laser).

Electrophysiology—Na+ transport was measured in FRT cells and in
H441 cells 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, 24 mM K2HPO4, 0.6 mM KH2PO4,
10 mM HEPES (pH 7.4) at 37 °C. Amiloride-sensitive short-circuit Na+
current (relative to 0 μM of Nedd4 or Nedd4-2) in FRT cells 48 h after
transfection with α-, β-, and γ-ENaC (0.067 μg of cDNA each), Nedd4
(C) or Nedd4-2 (D), and no siRNA (open circles), Nedd4 siRNA (closed
circles), or Nedd4-2 siRNA (closed squares) (0.1 μg) (mean ± S.E.,
n = 11–12). Asterisks indicate p < 5 × 10−4 versus no siRNA.

RESULTS

Selective Silencing of Nedd4 and Nedd4-2 with siRNAs—To
determine the role of Nedd4 and Nedd4-2 in ENaC regulation,
we generated siRNA duplexes to selectively reduce their
expression and used two independent assays to test siRNA activity
and specificity. First, we asked whether siRNAs would reduce the
expression of Nedd4 and Nedd4-2 proteins (GFP-tagged).
We cotransfected FRT cells with Nedd4-GFP and
Nedd4 siRNA, Nedd4-2 siRNA, or no siRNA and detected
Nedd4 protein by immunofluorescence (Fig. 1A, left). Nedd4
siRNA decreased the expression of Nedd4-GFP (compared with
no siRNA). In contrast, Nedd4-2 siRNA did not decrease
Nedd4-GFP protein. Conversely, in cells transfected with
Nedd4-2-GFP, Nedd4-2 siRNA decreased Nedd4-2-GFP protein,
but Nedd4 siRNA did not (Fig. 1A, right). The results in
H441 cells transfected with Nedd4-GFP or Nedd4-2-GFP were similar
(Fig. 1B).

Second, we used an electrophysiological assay to test whether siRNAs would prevent Nedd4 or Nedd4-2 from inhibit-
ing ENaC. We transfected FRT cells with α-, β-, and γ-ENaC
and measured transepithelial amiloride-sensitive Na+ current as an
assay of ENaC function. When cotransfected with ENaC,
Nedd4 and Nedd4-2 each decreased Na+ current in a dose-de-
dependent manner (Fig. 1, C and D). siRNA against Nedd4 abol-
hished the ability of Nedd4, but not Nedd4-2, to inhibit ENaC
(Fig. 1, C and D). Conversely, Nedd4-2 siRNA prevented
Nedd4-2, but not Nedd4, inhibition of ENaC (Fig. 1, C and D).
Together, the data indicate that siRNAs selectively reduce
expression of Nedd4 or Nedd4-2. This provides a strategy
to distinguish their relative contribution to ENaC regulation.

ENaC Regulation by Endogenous Nedd4 and Nedd4-2 in
Epithelia—If Nedd4 or Nedd4-2 is a physiological negative
regulator of ENaC under basal conditions, we predicted that
reducing its expression would increase ENaC Na+ current. We
tested this hypothesis using two epithelial cell lines: FRT cells,
which reconstitute critical aspects of ENaC regulation and
provide a useful model system (8, 10, 16, 24), and H441 cells, a
human lung epithelial cell line that transports Na+ across the
apical membrane. We cotransfected FRT cells with ENaC and siRNA against
Nedd4 or Nedd4-2. As negative controls, we used siRNA
against GFP or no siRNA. Nedd4 siRNA did not alter amilo-
ride-sensitive Na+ current compared with no siRNA or GFP
siRNA (Fig. 2B). In contrast, Nedd4-2 siRNA increased Na+
current (1.9-fold relative to no siRNA) (Fig. 2B). In H441 cells, we measured endogenous amiloride-sensitive Na\(^+\) current (Fig. 2C). Transfection with Nedd4-2 siRNA increased current (2.8-fold relative to GFP siRNA), but Nedd4 siRNA did not alter current. Together, the data suggest that endogenous Nedd4-2, but not Nedd4, negatively regulates ENaC in FRT and H441 epithelia.

Nedd4-2 inhibits ENaC through its binding to PY motifs located in the C termini of ENaC-subunits (9, 10). Because the PY motifs are deleted or mutated in Liddle’s syndrome, ENaC regulation by Nedd4-2 might be disrupted in this disorder. We therefore tested the hypothesis that a Liddle’s syndrome mutation would prevent Nedd4-2 siRNA from increasing Na\(^+\) current. FRT cells were transfected with wild-type \(\alpha\)- and \(\gamma\)-ENaC and a mutant \(\beta\)-subunit (\(\beta_{\text{Pybox}}\)) that lacks most of the C terminus including the PY motif. The cells were cotransfected with or without siRNA. Nedd4-2 siRNA did not increase Na\(^+\) current in cells transfected with the mutant ENaC (Fig. 2D) in contrast to our results with wild-type ENaC (Fig. 2B). Thus, an intact PY motif in \(\beta\)-ENaC is required for ENaC regulation by endogenous Nedd4-2.

Role of Nedd4-2 in Steroid Hormone Regulation of ENaC—Steroid hormones increase epithelial Na\(^+\) transport in part by inducing transcription of SGK. Previous work indicates that SGK phosphorylates Nedd4-2 and thereby decreases its interaction with ENaC (16, 17). Therefore, we tested the hypothesis that steroid hormones regulate Na\(^+\) transport, in part, through a pathway that includes Nedd4-2. We first asked whether Nedd4-2 depletion (by siRNA) would alter ENaC stimulation by dexamethasone. FRT cells transfected with ENaC were treated with or without dexamethasone (100 nM) for 6 h (following serum withdrawal for 24 h). Dexamethasone increased Na\(^+\) current in cells cotransfected with siRNA against GFP (1.0 \(\mu\)A/cm\(^2\)) or Nedd4 (0.9 \(\mu\)A/cm\(^2\)) (Fig. 3A, top panels). In contrast, dexamethasone failed to increase Na\(^+\) current in cells cotransfected with Nedd4-2 siRNA (Fig. 3A, top panels). This suggests that Nedd4-2 is required for dexamethasone to stimulate ENaC in FRT cells.

We asked whether Nedd4-2 also contributes to steroid hormone signaling in H441 epithelia. Dexamethasone (6 h) increased Na\(^+\) current in cells transfected with GFP siRNA or Nedd4 siRNA (4.3 and 3.7 \(\mu\)A/cm\(^2\), respectively) (Fig. 3A, bottom panels). In cells transfected with Nedd4-2 siRNA, dexamethasone produced a much smaller increase in Na\(^+\) current (1.6 \(\mu\)A/cm\(^2\)) (Fig. 3A, bottom panels). Thus, in H441 cells, Nedd4-2 contributes to steroid hormone regulation of endogenous ENaC.

As a reciprocal strategy, we overexpressed a constitutively active Nedd4-2 in FRT epithelia. SGK inhibits Nedd4-2 activity (shifting the Nedd4-2 dose-response relationship to the right) (Fig. 3B) by decreasing its binding to ENaC (16, 17). By mutating the three sites phosphorylated by SGK, we generated a Nedd4-2 construct (Nedd4-2S/T-A) that inhibited ENaC but could no longer be negatively regulated by SGK (Fig. 3C). We found that overexpression of Nedd4-2S/T-A nearly abolished the dexamethasone-mediated increase in Na\(^+\) current (0.1 \(\mu\)A/cm\(^2\)) (Fig. 3D). As a control, we overexpressed wild-type Nedd4-2, which did not prevent dexamethasone from increasing Na\(^+\) current (because it can be inhibited by SGK) (Fig. 3D). Together, data from these complementary approaches suggest that steroid hormones regulate ENaC in part by inhibiting Nedd4-2. Moreover, the data suggest that Nedd4-2 phosphorylation by SGK is a critical step in this signaling pathway.

DISCUSSION

Nedd4-2 and ENaC Regulation—The data provide direct evidence that HECT WW domain ubiquitin-protein ligases regulate ENaC in epithelia. Moreover, they define a specific regulatory role for Nedd4-2; silencing of Nedd4-2 increased Na\(^+\) current in two different epithelia, suggesting that endogenous Nedd4-2 inhibits ENaC in these cells. Conversely, the data suggest that Nedd4 does not regulate ENaC in these epithelia; silencing Nedd4 did not alter Na\(^+\) current. This was somewhat surprising because both Nedd4 and Nedd4-2 are expressed in a variety of Na\(^+\)-transporting epithelia (9, 10, 26), including the cells used for our study. Moreover, expressing either Nedd4 or...
ENaC Regulation by Nedd4-2

Fig. 3. Nedd4-2 and steroid hormones. A, amiloride-sensitive short-circuit Na⁺ current (left panels) and dexamethasone-induced increase in Na⁺ current (right panels) in FRT cells (top panels) and H441 cells (bottom panels) (µA/cm²; mean ± S.E.). Cells were cultured in serum-free medium ± dexamethasone (100 nm) for 6 h prior to study. FRT cells were transfected with ENaC (0.3 µg of each subunit) and siRNA (GFP, Nedd4, or Nedd4-2; 0.1 µg) (n = 14); asterisk indicates p < 6 × 10⁻⁴ versus control. H441 cells were transfected with GFP siRNA, Nedd4 siRNA, or Nedd4-2 siRNA (1 µg) (n = 9–11); asterisk indicates p < 0.03 versus control. Amiloride-sensitive short-circuit Na⁺ current (relative to 0 µg of Nedd4-2 or Nedd4-2 ΔS/T-A) in FRT cells transfected with ENaC (0.04 µg of cDNA each subunit), Nedd4-2 (B) or Nedd4-2 ΔS/T-A (C) (0–0.125 µg of cDNA), and SGK or an inactive SGK mutant (K127M, Control, 0.75 µg of cDNA) (n = 18); asterisks indicate p < 0.03. D, dexamethasone-induced increase in Na⁺ current (µA/cm²) in FRT cells transfected with ENaC (0.067 µg of each subunit) and GFP, Nedd4-2, or Nedd4-2 ΔS/T-A (0.8 µg of cDNA) (n = 16); asterisk indicates p < 5 × 10⁻⁴ versus GFP. E, model of ENaC regulation by steroid hormones.

Nedd4-2 in heterologous cells inhibits ENaC current (9, 13) (although compared with human Nedd4, mouse and rat Nedd4 have decreased potency because they lack WW domain 3 (8, 27)). It is possible that Nedd4 or other members of the Nedd4 family (e.g. WWP2) (10) inhibit ENaC in different epithelia and contribute to tissue-specific differences in ENaC regulation.

Nedd4-2 and Steroid Hormones—The data also support a role for Nedd4-2 in ENaC regulation by steroid hormones; Nedd4-2 siRNA reduced ENaC stimulation by dexamethasone in FRT and H441 epithelia. This was specific to Nedd4-2, consistent with the lack of SGK phosphorylation consensus sites in Nedd4 (and other Nedd4 family members). Together with previous work, the data suggest the model illustrated in Fig. 3E. Under basal conditions, Nedd4-2 is unphosphorylated, allowing it to bind to ENaC PY motifs and to target the channel for degradation. As a result, Nedd4-2 minimizes epithelial Na⁺ transport. Conversely, in response to Na⁺ deprivation, steroid hormones increase expression of SGK, which phosphorylates Nedd4-2. In the phosphorylated state, Nedd4-2 binding to ENaC is reduced, stabilizing channel complexes at the cell surface. As a result, steroid hormones increase epithelial Na⁺ absorption.

What is the relative contribution of this SGK/Nedd4-2 pathway to ENaC regulation by steroid hormones? Our data suggest that it plays an important role; Nedd4-2 siRNA abolished the response to dexamethasone in FRT cells and reduced it substantially in H441 cells. However, it is clear that additional pathways also contribute. First, in H441 cells, Nedd4-2 siRNA did not completely abolish ENaC regulation by dexamethasone (although we cannot exclude the possibility that Nedd4-2 expression was not completely silenced). Second, there is compelling evidence from previous studies that steroid hormones increase ENaC transcription, likely contributing to the “late” (6–24 h) increase in Na⁺ absorption (Fig. 3E) (28, 29). It is likely that additional steroid-induced genes also contribute to ENaC regulation, including K-Ras2A, a small G protein (30). Third, two recent studies found that mutation of ENaC PY motifs did not abolish ENaC regulation by steroid hormones in mouse collecting duct epithelia (31, 32). However, a limitation of these studies is that the PY motifs were not mutated in all three subunits; Nedd4-2 is capable of inhibiting ENaC when only a single PY motif is intact (9).

Nedd4-2 and Liddle’s Syndrome—Two potential mechanisms have been proposed to explain the excessive renal Na⁺ absorption that causes hypertension in Liddle’s syndrome. First, the sequence disrupted by Liddle’s syndrome mutations (PPPXYXXX) fits the consensus of a PY motif (PPXYP) (Φ indicates hydrophobic residue) (11, 33). Thus, Liddle’s syndrome might result from a defect in ENaC regulation by Nedd4 family members. Second, the disrupted sequence is also similar to clathrin-mediated endocytosis motifs (e.g. NPYXY, YXXΦ) (Φ indicates hydrophobic residue) (11, 33). Thus, Liddle’s syndrome might result from a defect in ENaC endocytosis. Both mechanisms could explain the increase in ENaC surface expression that causes excessive epithelial Na⁺ absorption in this disorder.

In the current studies, we found that Nedd4-2 depletion (by siRNA) mimicked the effect of Liddle’s syndrome mutations by increasing Na⁺ current. Moreover, a Liddle’s syndrome mutation in β-ENaC prevented a further increase in Na⁺ current in response to Nedd4-2 depletion. Further, the data suggest that Nedd4-2 and Liddle’s syndrome mutations alter Na⁺ current through a common pathway, supporting the hypothesis that Liddle’s syndrome is caused at least in part by a defect in ENaC regulation by Nedd4-2. In addition, it raises the intriguing possibility that loss-of-function mutations in Nedd4-2 might contribute to the pathogenesis of hypertension in some populations.

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