Inhibition of the Epithelial Na\textsuperscript{+} Channel by Interaction of Nedd4 with a PY Motif Deleted in Liddle’s Syndrome*

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The epithelial Na\textsuperscript{+} channel (ENaC) plays a critical role in Na\textsuperscript{+} absorption in the kidney and other epithelia. Mutations in the C terminus of the \( \beta \) or \( \gamma \) ENaC subunits increase renal Na\textsuperscript{+} absorption, causing Liddle’s syndrome, an inherited form of hypertension. These mutations delete or disrupt a PY motif that was recently shown to interact with Nedd4, a ubiquitin-protein ligase expressed in epithelia. We found that Nedd4 inhibited ENaC when they were coexpressed in Xenopus oocytes. Liddle’s syndrome-associated mutations that prevent the interaction between Nedd4 and ENaC abolished inhibition, suggesting that a direct interaction is required for inhibition by Nedd4. Inhibition also required activity of a ubiquitin ligase domain within the C terminus of Nedd4. Nedd4 had no detectable effect on the single channel properties of ENaC. Rather, Nedd4 decreased cell surface expression of both ENaC and a chimeric protein containing the C terminus of the \( \beta \) subunit. Decreased surface expression resulted from an increase in the rate of degradation of the channel complex. Thus, interaction of Nedd4 with the C terminus of ENaC inhibits Na\textsuperscript{+} absorption, and loss of this interaction may play a role in the pathogenesis of Liddle’s syndrome and other forms of hypertension.

The epithelial Na\textsuperscript{+} channel (ENaC)\textsuperscript{1} is expressed at the apical membrane of a variety of epithelia, including the kidney, lung, and colon (1, 2). Because Na\textsuperscript{+} entry through ENaC is the rate-limiting step for Na\textsuperscript{+} absorption, regulation of this channel plays a critical role in controlling extracellular fluid volume and blood pressure. The channel consists of three homologous subunits (\( \alpha, \beta, \) and \( \gamma \)) (3–7). Mutations that truncate the cytoplasmic C terminus of the \( \beta \) or \( \gamma \) subunits increase Na\textsuperscript{+} absorption in the kidney collecting duct, resulting in Liddle’s syndrome, an autosomal dominant form of hypertension (8, 9). Previous studies found that Liddle’s syndrome-associated mutations increased Na\textsuperscript{+} current compared with expression of ENaC alone. One to two days after injection, whole cell current was measured by two-electrode voltage-clamp.

However, direct evidence to support this hypothesis is lacking. Thus, it is tempting to speculate that Nedd4 inhibits Na\textsuperscript{+} absorption through ENaC and that loss of this inhibition plays a role in Liddle’s syndrome. However, direct evidence to support this hypothesis is lacking. Here we coexpress Nedd4 with ENaC to directly test the hypothesis that Nedd4 inhibits ENaC, demonstrating the functional importance of this WW domain-PY motif interaction.

EXPERIMENTAL PROCEDURES

DNA Constructs—Nedd4 (rat) was cloned by polymerase chain reaction of cDNA reverse transcribed from rat lung poly(A)\textsuperscript{+} RNA (CLONTECH). Two independent clones were generated that produced identical functional results. A Nedd4 construct containing only the three WW domains (Nedd4\textsubscript{WW}) was generated by polymerase chain reaction (amino acids 233–500). Mutation of Cyg\textsuperscript{X} (X = alanine (Nedd4\textsubscript{WXXA}) did not alter Na\textsuperscript{+} current compared with expression of ENaC alone. One to two days after injection, whole cell current was measured by two-electrode voltage-clamp.

The abbreviations used are: ENaC, epithelial Na\textsuperscript{+} channel; SEAP, secreted alkaline phosphatase; MTS, methanethiosulfonate.

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PPYYXXL. Two findings suggest that this tyrosine-based sequence plays an important role in controlling Na\textsuperscript{+} absorption; missense mutations in this sequence increased Na\textsuperscript{+} current similar to the C-terminal truncations (11, 12), and mutations in this sequence have been identified in families with Liddle’s syndrome (13, 14). Deletion or disruption of the tyrosine-based sequence increased Na\textsuperscript{+} current at least in part by increasing the number of Na\textsuperscript{+} channels at the cell surface (10, 11). Although it is not yet known how these mutations increase cell surface expression, similarity of the sequence to previously described protein motifs suggest two potential mechanisms.

First, the PPPYYXXL sequence is similar to two different tyrosine-based internalization motifs found in proteins such as the LDL receptor (NP\textsubscript{XYY}) (15) and the transferrin receptor (XXYL/hydrophobic) (16). If this sequence functions as an internalization signal in ENaC, then Liddle’s mutations might lead to an accumulation of ENaC at the cell surface by disrupting channel internalization.

Second, the tyrosine-based sequence fits the consensus of a recently defined motif that plays a role in protein-protein interactions, the PY motif (PPPY) (17). PY motifs were identified by their ability to bind to a 35–40- amino acid sequence containing two conserved tryptophan residues (WW domain) (17). Although the functional effects of these interactions are not yet known, this observation suggests that an interacting protein might control the surface expression of ENaC. A candidate is Nedd4, a protein expressed in epithelia that contains three WW domains (18–20). Using a biochemical assay, Staub and co-workers found that Nedd4 interacted with the PY motifs of ENaC (21). In addition, mutations that cause Liddle’s syndrome abolished this interaction. Thus, it is tempting to speculate that Nedd4 inhibits Na\textsuperscript{+} absorption through ENaC and that loss of this inhibition plays a role in Liddle’s syndrome. However, direct evidence to support this hypothesis is lacking. Here we coexpress Nedd4 with ENaC to directly test the hypothesis that Nedd4 inhibits ENaC, demonstrating the functional importance of this WW domain-PY motif interaction.

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clamp at −60 mV with oocytes bathed in 116 mM NaCl, 2 mM KCl, 0.4 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES (pH 7.4). Single channel currents were recorded by cell-attached patch-clamp in devitellinized oocytes one day after injection as described previously (11). The extra-cellular solution was identical to that described above, and LiCl replaced NaCl in the pipette solution. Statistical significance was assessed using a Student's unpaired t test.

Surface Expression of ENaC in Xenopus Oocytes—αENaC, βENaC, and γENaC (22) were coexpressed (ENαCγ) in Xenopus oocytes (0.2 ng each) with either Nedd4 or SEAP (0.8 ng). As a negative control, we injected oocytes with the vector (pMT3) alone. 1 h after injection, we treated oocytes with 1 mM (2-aminoethyl)methanethiosulfonate hydrobromide (Toronto Research Chemicals) for 10 min at room temperature to modify endogenous sulfhydryls. Similar results were also obtained using 2-(trithyiaminomethyl)ethyl methanethiosulfonate bromide. 16–20 h after injection, oocytes were placed on ice, labeled with 2 [(5-fluoresceinyl)aminoacarbonyl] ethyl methanethiosulfonate (MTS-4-fluorescein, Toronto Research Chemicals) (200 µM in modified Barth solution) for 5 min, and washed for 15 min three times with cold modified Barth solution. MTS-4-fluorescein irreversibly stimulates ENαCγ, but not wild-type ENaC. Nedd4 did not alter the ability of MTS-4-fluorescein to stimulate ENαCγ, suggesting that Nedd4 did not alter accessibility of the introduced cysteines. Cell surface fluorescence was quantitated by confocal microscopy (Bio-Rad MRC-600, krypton/argon laser). Optical sections obtained at 30-µm steps were superimposed, mean fluorescence at the plasma membrane was determined, and background (pMT3 alone) was subtracted.

Biotinylation of A2-β Chimeras—Chimeras containing the extracellular and transmembrane segments of the HLA protein A2 and the intracellular C terminus of βENaC (wild type or Y620A) were constructed in pcDNA3 and expressed in COS-7 cells by electroporation (with 10 µg of Nedd4 or SEAP) as described previously (11). One to two days after electroporation, cells were biotinylated with sulfo-succinimidyl-6-(biotinamido)-6- hexamido hexaoxate (0.5 mg/ml, Pierce) in phosphate-buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂ at 4 °C for 30 min. Cells were washed three times, solubilized in cold lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4) containing 1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 20 µg/ml leupeptin, and 10 µg/ml pepstatin A, and cleared by centrifugation at 14,000 rpm for 10 min. A2-β in the supernatant was immunoprecipitated with an anti-A2 monoclonal antibody. Biotinylated A2-β was detected using the ABC method (Vectastain) and enhanced chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech) and quantified by phosphorimaging (Storm with ImageQuant software, Molecular Dynamics).

Expression of ENaC with Nedd4 in COS-7 Cells—COS-7 cells were electroporated with α, β, and γENaC (10 µg each) with or without Nedd4 (10 µg), pulse-labeled with 100 µCi/ml [35S]methionine (New England Nuclear) at 37 °C for 30 min, and then chased for 0–4 h. Proteins were solubilized in cold lysis buffer containing 1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 20 µg/ml leupeptin, and 10 µg/ml pepstatin A. Protein insoluble to Triton X-100 was solubilized in 2% SDS at 95 °C and then diluted 10-fold in lysis buffer containing 1% Triton X-100. αENaC in the Triton X-100 soluble and insoluble fractions was immunoprecipitated with anti-Flag M2 antibody (Eastman Kodak), which recognized a Flag epitope in the extracellular domain, as described previously (11). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and imaged and quantitated by phosphorimaging.

RESULTS
Inhibition of ENaC by Nedd4—To test the hypothesis that Nedd4 inhibits ENaC Na⁺ current, we coexpressed Nedd4 with ENaC in Xenopus oocytes and measured the whole cell Na⁺ current blocked by amiloride. When we injected equal amounts of cDNA encoding Nedd4 and each ENaC subunit, Nedd4 decreased amiloride-sensitive Na⁺ current 55% (Fig. 1, A–C). Injection of a 4-fold higher amount of Nedd4 cDNA decreased current 88% (Fig. 1B). In contrast to its effect on Na⁺ current, Nedd4 did not alter several characteristic biophysical properties of ENaC, including its voltage independence (Fig. 1C), its high selectivity for Na⁺ over K⁺, and its sensitivity to amiloride (not shown).

Biochemical studies showed that Nedd4 binds to the PY motif in the C terminus of ENaC subunits (21). The Liddle’s syndrome mutation R566X deletes most of the cytoplasmic C terminus of the β subunit, including the PY motif. We therefore asked whether this mutation would disrupt inhibition by Nedd4. Coexpression of wild-type α and γENaC with βR566X increased Na⁺ current compared with wild-type β (Fig. 2), as previously reported (10, 11). However, Nedd4 did not inhibit the mutant channel, suggesting that the C terminus of βENaC was required for inhibition by Nedd4. Mutation of Tyr²⁰⁰ in the PY motif to alanine also increased Na⁺ current and disrupted inhibition by Nedd4 (Fig. 2). This tyrosine is required for WW domain-PY motif interactions (17), and an equivalent mutation in a peptide derived from the PYY motif of βENaC abolished binding of the peptide to Nedd4 (21). These data suggest that a direct interaction between Nedd4 and the PY motif in βENaC is required for channel inhibition by Nedd4.

Role of Nedd4 Ubiquitin Ligase Domain—Although the WW domains of Nedd4 are sufficient for binding to the C-terminal PY motifs of ENaC (21), Nedd4 also contains a calcium phospholipid binding domain (C2 domain (23)) and a ubiquitin ligase domain (20, 21). To determine whether these domains are required for inhibition by Nedd4, we tested a construct containing only the three WW domains (Nedd4WW). In contrast to wild-type Nedd4, expression of Nedd4WW with ENaC did not decrease Na⁺ current (Fig. 3A). Thus, binding of the WW domains to the PY motif alone is not sufficient to inhibit ENaC; this suggests that the C2 domain, ubiquitin ligase domain, or both are also required.

Ubiquitin ligase domains transfer ubiquitin to target substrates. A conserved cysteine within the domain forms a thioester bond with ubiquitin, and mutation of this cysteine abolishes its ability to accept ubiquitin (24). To test the hypothesis that the ubiquitin ligase domain is required for ENaC inhibition by Nedd4, we mutated the conserved cysteine to alanine.
This mutation disrupted inhibition of ENaC by Nedd4; expression of equal amounts of cDNA encoding Nedd4C854A and each ENaC subunit did not significantly decrease Na\(^+\) current (Fig. 3B). A 4-fold higher amount of Nedd4C854A cDNA decreased Na\(^+\) current 33% (Fig. 3B), although this was significantly less than the decrease produced by wild-type Nedd4. Therefore, the ubiquitin ligase domain and Cys854 play an important role in the inhibition of ENaC by Nedd4. However, the small amount of inhibition produced by higher concentrations of Nedd4C854A indicates that the mutant has partial activity. Thus, part of the inhibitory effect of Nedd4 may not require its ubiquitin ligase activity.

Single Channel Properties of ENaC Coexpressed with Nedd4—Nedd4 could inhibit ENaC by decreasing open-channel probability (\(P_o\)), single channel conductance, or the number of channels at the cell surface. To test the first two possibilities, we measured the single channel properties of ENaC expressed with Nedd4 in \textit{Xenopus} oocytes. ENaC is characterized by very slow kinetics, with long channel openings and closings (2, 5, 11). Fig. 4A shows a patch containing a single channel from a cell coexpressing ENaC and Nedd4. Nedd4 did not alter the slow kinetics of ENaC or produce a detectable decrease in \(P_o\) (Fig. 4B). Fig. 4C shows the single channel current-voltage plot (mean ± S.E., \(n = 19–25\)). Single channel conductance was \(6.19 ± 0.16\) picosiemens for \(2\) Nedd4 and \(6.29 ± 0.10\) picosiemens for +Nedd4 (\(p = 0.62\)).

FIG. 2. Deletion or mutation of PY motif in \(\beta\)ENaC. Amiloride-sensitive Na\(^+\) current (relative to wild-type ENaC) in oocytes coexpressing Nedd4 or control plasmid (0.2 ng) with \(\alpha\), \(\gamma\), and wild-type (WT) or mutant \(\beta\)ENaC (R566X or Y620A) as indicated (0.2 ng each). Data are the means ± S.E. for \(n = 40–53\). The asterisk indicates \(p < 0.0001\). Nedd4 did not significantly decrease Na\(^+\) current for R566X and Y620A.

FIG. 3. Coexpression of ENaC with Nedd4 constructs. A, Amiloride-sensitive Na\(^+\) current (relative to –Nedd4) for ENaC coexpressed in oocytes with SEAP (–Nedd4), Nedd4, or Nedd4\(_{ww}\) (mean ± S.E., \(n = 34–62\)). Nedd4\(_{ww}\) and –Nedd4 were not statistically different. B, amiloride-sensitive Na\(^+\) current (relative to –Nedd4) for ENaC coexpressed with SEAP (–Nedd4, black bars), Nedd4 (hatched bars), or Nedd4\(_{C854A}\) (white bars) (0.2 or 0.8 ng, as indicated). Data are the means ± S.E. for \(n = 10–17\). * values are indicated.

FIG. 4. Single channel properties of ENaC coexpressed with Nedd4. A, representative current tracing of ENaC expressed in oocyte with Nedd4 (0.2 ng each) in cell-attached configuration at \(-60\) mV. Patch contains a single channel; open and closed states are indicated. B, \(P_o\) for ENaC expressed with or without Nedd4 (mean ± S.E., \(n = 9–10\), \(p = 0.45\)). Because the \(P_o\) of ENaC is highly variable, we cannot completely exclude an effect of Nedd4 on \(P_o\). C, single channel current-voltage plot (mean ± S.E., \(n = 19–25\)). Single channel conductance was \(6.19 ± 0.16\) picosiemens for –Nedd4 and \(6.29 ± 0.10\) picosiemens for +Nedd4 (\(p = 0.62\)).
quantitated ENaC at the cell surface using confocal microscopy, similar to a method previously described (27). To minimize background, we used a nonfluorescent MTS compound ((2-aminoethyl)methanethiosulfonate hydrobromide) to modify cysteines in endogenous cell surface proteins 1 h after cDNA injection (16–20 h before study). Oocytes expressing the cysteine-tagged ENaC (ENaCCys) had increased cell surface fluorescence compared with oocytes injected with vector cDNA as indicated. A, optical sections of oocytes labeled with MTS-4-fluorescein were obtained by confocal microscopy and superimposed (13 sections at 30-μm steps). The images show fluorescence at the cell surface. B, quantitation of cell surface fluorescence. Data are arbitrary fluorescence units (mean ± S.E., n = 8–30) corrected for background. The asterisk indicates p < 0.03.

**Fig. 5.** Nedd4 binding decreases cell surface expression of ENaC. Cell surface fluorescence in oocytes injected with cDNA encoding pMT3 alone, ENaCCys, or ENaCCys-Y620A with either Nedd4 (+Nedd4) or SEAP (−Nedd4) (0.8 ng) as indicated. A, optical sections of oocytes labeled with MTS-4-fluorescein were obtained by confocal microscopy and superimposed (13 sections at 30-μm steps). The images show fluorescence at the cell surface. B, quantitation of cell surface fluorescence. Data are the means ± S.E. (n = 5 for each), and the asterisk indicates p < 0.025.

**Fig. 6.** Nedd4 decreases cell surface expression of a chimeric protein containing the C terminus of βENaC. A, schematic representation of A2-β chimeric protein. The site of Y620A mutation is indicated (numbering refers to residue number within βENaC). B and C, Western blots and quantitation (relative to −Nedd4) of biotinylated A2-β (B) or A2-β-Y620A (C) expressed in COS-7 cells with or without Nedd4 as indicated. COS-7 cells were biotinylated or not sulfosuccinimidyl-6′-(biotinamido)-6-hexamido hexamethoazote as indicated. Data are the means ± S.E. (n = 5 for each), and the asterisk indicates p < 0.025.

Cellular and membrane-spanning domains of A2, an HLA protein, to the cytoplasmic C terminus of βENaC (A2-β, Fig. 6A). Following expression in COS-7 cells, we biotinylated cell surface proteins and detected and quantitated biotinylated A2-β as an assay of its surface expression. Expression of A2-β produced a 55-kDa biotinylated band (Fig. 6B) corresponding to the expected size of A2-β (11). This band was not seen in cells that were not biotinylated (Fig. 6B) or in untransfected cells (not shown). Coexpression with Nedd4 produced a significant decrease in biotinylated A2-β, indicating that Nedd4 decreased the amount of A2-β at the cell surface (Fig. 6B). Thus, interaction of Nedd4 with the C-terminal PY motif was sufficient to decrease surface expression of an unrelated protein.

**Decreased Stability of ENaC Expressed with Nedd4 in COS-7 Cells**—Our finding that Nedd4 decreased the number of ENaC channels at the cell surface, coupled with the requirement for the ubiquitin ligase activity of Nedd4, suggests that Nedd4 might target ENaC for degradation. To test this hypothesis, we coexpressed α, β, and γENaC in COS-7 cells with or without Nedd4. Following pulse labeling with [35S]methionine (0 h chase) and solubilization with Triton X-100, we immunoprecipitated the α subunit as a marker for the channel complex. This produced two predominant bands corresponding to the unglycosylated and glycosylated forms of αENaC (Fig. 7A). When we coexpressed ENaC with Nedd4, an additional band was observed, corresponding to Nedd4 that coprecipitated with ENaC. Nedd4 did not decrease the amount of 35S-labeled αENaC immediately after pulse labeling, suggesting that Nedd4 did not decrease the rate of synthesis of αENaC. In contrast, Nedd4 decreased the amount of αENaC protein dur-
Nedd4 Decreases Surface Expression of ENaC

The binding of Nedd4 to a subsequent degradation of the subunit. In ubiquitination, internalization, and single ENaC subunit is shown, resulting in the cell surface expression of ENaC. Nedd4 interacts with ENaC through the binding of its WW domains to PY motifs in the C terminus of ENaC. This interaction is required for Nedd4 to inhibit ENaC; mutations that abolished Nedd4 binding prevented inhibition of ENaC by Nedd4. The PY motif-WW domain interaction may provide specificity, targeting the activity of Nedd4 to ENaC and possibly other proteins containing PY motifs. However, interaction between ENaC and the WW domains alone was not sufficient to inhibit ENaC, suggesting that other sequences within Nedd4 are required for decreased surface expression.

Several observations suggest that the ubiquitin ligase domain of Nedd4 plays an important role in the inhibition of ENaC. First, this domain is homologous to E3 ubiquitin-protein ligases, which transfer ubiquitin to target proteins (24), indicating other proteins containing PY motifs. However, interaction between ENaC and the WW domains alone was not sufficient to inhibit ENaC, suggesting that other sequences within Nedd4 are required for decreased surface expression.

Several observations suggest that the ubiquitin ligase domain of Nedd4 plays an important role in the inhibition of ENaC. First, this domain is homologous to E3 ubiquitin-protein ligases, which transfer ubiquitin to target proteins (24), increasing their rate of degradation. Recent work indicates that Nedd4 has ubiquitin ligase enzymatic activity (28). Second, it was recently reported that lysines within the N termini of α and γENaC are substrates for ubiquitination and that mutation of these lysines increased ENaC Na⁺ current in Xenopus oocytes (29). However, the role of Nedd4 in ubiquitination of these lysines is unknown. Finally, we found that mutation of a residue in Nedd4 required for ubiquitin conjugation (Cys854) significantly decreased the ability of Nedd4 to inhibit ENaC. This suggests a model in which the WW domain-PY motif interaction directs the ubiquitin ligase domain to the channel, where it can ubiquitinate one or more subunit(s) to target the complex for degradation (Fig. 8). Consistent with this model, we found that Nedd4 increased the rate of degradation of ENaC. It is also possible that Nedd4 interacts with ENaC in an intracellular compartment, preventing transport of the channel complex to the cell surface.

Nedd4 might also decrease ENaC at the cell surface by increasing the rate of channel internalization by two potential mechanisms. First, ubiquitination by Nedd4 might stimulate channel internalization (Fig. 8); ubiquitination was recently shown to be required for internalization of the yeast pheromone receptor Ste2p (30). Second, the Nedd4 C2 domain could play a role in the internalization of ENaC; a C2 domain within synaptotagmin I mediates endocytosis of synaptic vesicles by binding to the clathrin AP-2 complex (31). Our finding that Nedd4C854A retained partial activity supports such a functional role for the C2 domain in the inhibition of ENaC. A recent report that a dominant negative dynamin increased ENaC current in Xenopus oocytes (32) also supports a role for endocytosis in controlling Na⁺ absorption. Although our results indicate that the PPPXYXYYL sequence functions as a PY motif, similarity of this sequence to tyrosine-based internalization motifs suggests that it could also play a role in ENaC internalization independent of its function as a PY motif (11).

A recent report by Dinudom et al. (33) suggests that Nedd4 or a related WW domain protein may be involved in inhibition of Na⁺ current in response to high levels of intracellular Na⁺ in mouse mandibular salivary duct cells. They found that a WW domain peptide or a polyclonal antibody against Nedd4 disrupted inhibition of Na⁺ current in response to 72 mM intracellular Na⁺. A dominant-negative ubiquitin also disrupted inhibition by high Na⁺, consistent with our finding that the ubiquitin ligase activity of Nedd4 plays an important role in its ability to inhibit ENaC. However, it is not known whether ENaC is responsible for Na⁺ current in mouse mandibular salivary duct cells, and the molecular identity of the WW domain protein involved in inhibition is also unknown. Interestingly, a number of other WW domain-containing proteins have been identified (20, 34), including several with significant homology to Nedd4, and some have been shown to bind to the PY motifs of ENaC (34). Thus, the WW domain might serve as an adapter to allow a variety of regulatory proteins with diverse functions to bind to the C terminus of ENaC, regulating channel function and, hence, Na⁺ absorption.

Nedd4 contains three WW domains, and the α, β, and γENaC

![Fig. 7. Nedd4 decreases stability of ENaC. α, β, and γENaC were expressed with or without Nedd4 (as indicated) in COS-7 cells, pulse labeled with [35S]methionine, chased for 0–4 h, and solubilized with Triton X-100, and αENaC was immunoprecipitated. A, immunoprecipitation immediately after pulse labeling. αENaC and Nedd4 are indicated. At later time points, βENaC that coprecipitated with αENaC could also be identified on the gel based on its different mobility. γENaC could not be detected because its migration is similar to that of αENaC. B, quantitation of total αENaC (Triton X-100 soluble and insoluble) by phosphorimaging (relative to t = 0) at 0–4 h chase. Similar results were obtained in three experiments.](image)

![Fig. 8. Model of inhibition of ENaC by Nedd4. The binding of Nedd4 to a single ENaC subunit is shown, resulting in ubiquitination, internalization, and subsequent degradation of the subunit.](image)
subunits each contain a PY motif. It therefore seems likely that Nedd4 binds to more than one subunit within a channel complex. Alternatively, Nedd4 might cluster channels by binding to PY motifs from two or three different channels. Interestingly, we found that mutation or deletion of the βENaC PY motif prevented inhibition by Nedd4, suggesting that interaction with the β subunit is required for channel inhibition. One interpretation of this result is that the β subunit is the functionally relevant substrate for Nedd4. Alternatively, the functional interaction between Nedd4 and ENaC might require a specific pattern of binding to PY motifs in more than one subunit.

In Liddle’s syndrome, deletion or mutation of conserved amino acids in the PY motif in ENaC increases Na⁺ absorption, resulting in hypertension. Because Liddle’s syndrome-associated mutations abolished binding and inhibition of ENaC by Nedd4, our data suggest that loss of control of ENaC surface expression by Nedd4 might be involved in the pathophysiology of Liddle’s syndrome. It seems possible that loss of function mutations in Nedd4 could also underlie other forms of hypertension. Thus, an understanding of the control of ENaC by Nedd4 and other related proteins may provide new insight into electrolyte homeostasis and the pathophysiology of hypertension.

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